

ANNUAL REVIEW OF BIOCHEMISTRY

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PREFACE

A preface may fulfill many functions. Lermontov pointed out long ago that a preface should be an apology for the book and a reply to its critics. In this vein, we must express our regrets to our authors for obliging them, through restrictions on space, to encompass so much in so little. We do not apologize to our readers for the book itself unless it be for our temerity in planning a review of the whole of a year's contributions to biochemistry in a single volume—even a big one. The solution of this problem, fast increasing in gravity, has us baffled. Shall we recommend that the volume be fattened even more and be increased in price? Shall we omit a few "standard" topics? Shall we retain the usual twenty or so and enforce even more painfully the present distressing restrictions on space?

The critics have given us too little to which to reply. They have been all too quiet for our own good. In this volume we hope that we have been sufficiently provocative to stir them into action. Impressed by the well-considered case put forth by Malcolm Dixon (*Nature and Science*, November, 1960) for abandonment of DPN and TPN, we have parted company with these old friends and accepted instead their more attractive cousins NAD (nicotinamide-adenine dinucleotide) and NADP (nicotinamide-adenine dinucleotide phosphate). These, in fact, are the recommendations of the Enzyme Commission of the international Union of Biochemistry and are expected, in the course of time, to receive definitive adoption by both the I.U.B. and the International Union of Pure and Applied Chemistry.

It is not possible, however, to implement these specific recommendations without going somewhat further. In casting off DPN and TPN, we must simultaneously discard DPNH and TPNH. On the assumption that a terminal H_2 is an acceptable symbolic additive, we have used throughout this volume $NADH_2$ and $NADPH_2$ to designate the reduced forms of NAD and NADP. The comments of the critics are now invited.

There are many who have contributed to this volume. To the authors we are especially indebted. We know something of the labors that were theirs. The thanks that we wish to convey are not only our own but, we believe, those of the readers as well. We wish also to express our appreciation to Floyd Daft who for five years has helped us with his advice as a member of the Editorial Committee. We welcome, as his successor, Alton Meister who will serve through 1965.

The subject index was prepared by James P. Scannell of Southern Illinois University. To him we are very grateful indeed. Our very warm thanks are also extended to our assistant editors, in particular Betty Schink who has borne much of the responsibility for seeing this volume through the press. Finally we owe much to the George Banta Company, who for many years has been our printer.

F.W.A.	G.M.
H.E.C. 1	E.L.S.
J.M.L. 1	E.E.S.
E.S.	

ERRATA

Volume 29 (1960):

page 244, line 14: *for Hsien et al. (382 to 384) read Wu et al. (382 to 384)*

page 258, references 382 to 384: *for Hsien, W. read Wu, H.*

page 725, third column, last line: *delete Hsien, W., 244*

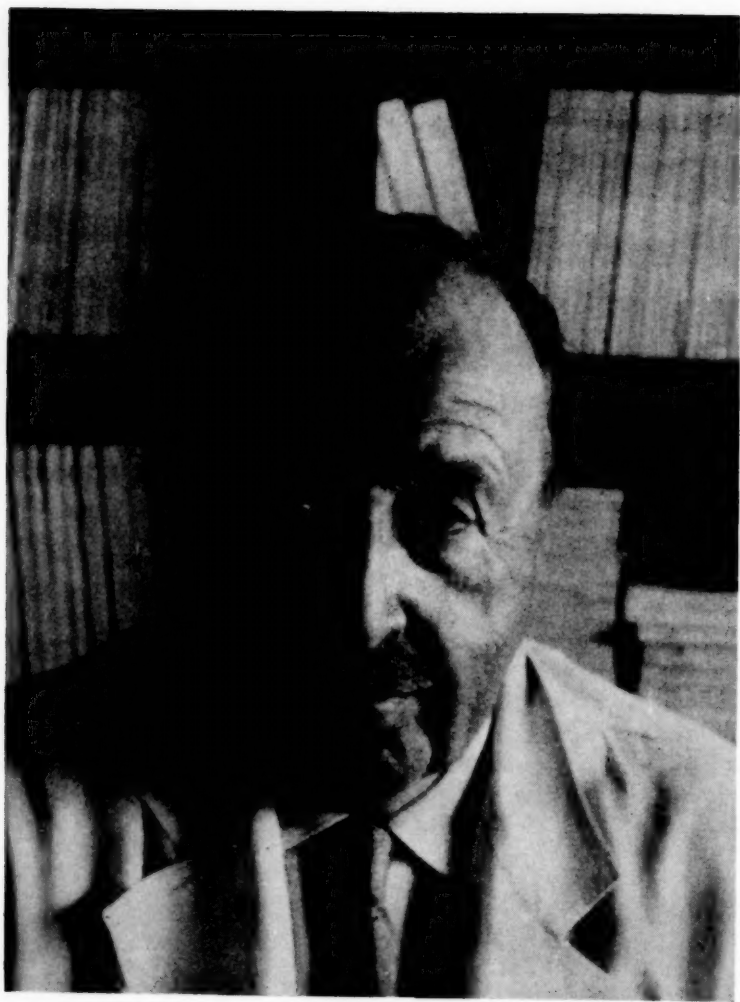
page 747, second column, between lines 51 and 52: *insert Wu, H., 244*

CONTENTS

PREFATORY CHAPTER—BIOCHEMISTS, BIOLOGISTS, AND WILLIAM OF OCCAM, <i>J. H. Northrop</i>	1
BIOLOGICAL OXIDATION, <i>M. Morrison</i>	11
CHEMISTRY OF THE CARBOHYDRATES, <i>A. B. Foster</i>	45
CHEMISTRY OF LIPIDS, <i>J. Asselineau and E. Lederer</i>	71
X-RAY STUDIES OF COMPOUNDS OF BIOLOGICAL INTEREST, <i>A. Rich and D. W. Green</i>	93
CHEMISTRY OF THE NUCLEOTIDES, <i>A. M. Michelson</i>	133
NUCLEIC ACID METABOLISM AND BIOSYNTHESIS, <i>R. Abrams</i>	165
LIPID METABOLISM, <i>R. G. Langdon and A. H. Phillips</i>	189
CARBOHYDRATE METABOLISM, <i>A. Sols</i>	213
METABOLISM OF AMINO ACIDS, <i>C. Gilvarg</i>	239
THE STRUCTURE OF PROTEINS, <i>B. S. Harrap, W. B. Gratzer, and P. Doty</i>	269
SPECIFICITY IN PROTEIN SYNTHESIS, <i>P. Berg</i>	293
PROTEIN NUTRITION, <i>W. N. Pearson and W. J. Darby</i>	325
GLYCOSIDASES AND TRANSGLYCOSIDATION, <i>R. A. Dedonder</i>	347
WATER-SOLUBLE VITAMINS, PART I, <i>A. E. Axelrod and C. J. Martin</i>	383
WATER-SOLUBLE VITAMINS, PART II, <i>G. W. E. Plaut</i>	409
FAT-SOLUBLE VITAMINS, <i>F. Bro-Rasmussen and W. Hjarde</i>	447
THE BIOCHEMISTRY OF MUSCLE, <i>S. V. Perry</i>	473
BIOCHEMISTRY OF STEROID HORMONES, <i>L. L. Engel and L. J. Langer</i>	499
BIOSYNTHESIS OF ISOPRENOID COMPOUNDS, <i>L. D. Wright</i>	525
PORPHYRINS AND HEMOPROTEINS, <i>E. Margoliash</i>	549
THE BIOCHEMISTRY OF THE POLYAMINES—SPERMIDINE AND SPERMINE, <i>H. Tabor, C. W. Tabor, and S. M. Rosenthal</i>	579
BIOCHEMISTRY OF CULTURED MAMMALIAN CELLS, <i>L. Levintow and H. Eagle</i>	605
BIOCHEMISTRY OF GENETIC FACTORS, <i>C. Levinthal and P. F. Davison</i>	641
BIOCHEMISTRY OF THE DIVIDING CELL, <i>D. Mazia</i>	669
OTHER REVIEWS OF BIOCHEMICAL INTEREST	689
INDEXES	691
AUTHOR INDEX	
SUBJECT INDEX	
CUMULATIVE INDEX OF CHAPTER TITLES, VOLUMES 26 TO 30	
CUMULATIVE INDEX OF CONTRIBUTING AUTHORS, VOLUMES 26 TO 30	

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John H. Hartung

PREFATORY CHAPTER

BIOCHEMISTS, BIOLOGISTS, AND WILLIAM OF OCCAM¹

BY JOHN H. NORTHROP

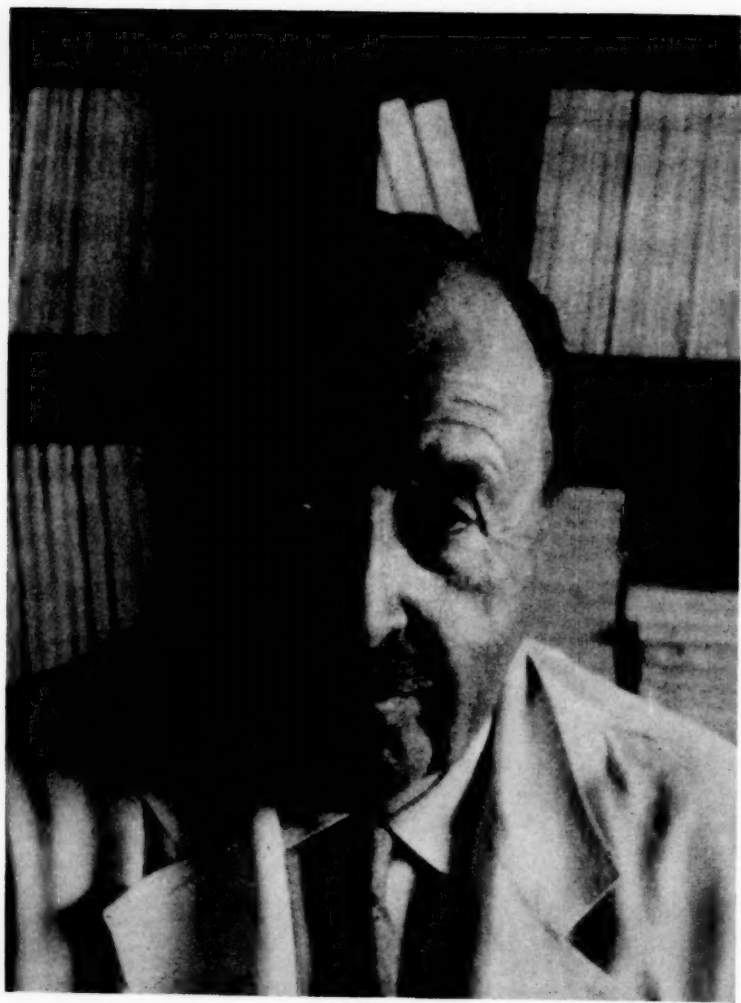
*Professor and Member of the Rockefeller Institute, New York, New York; Research
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The biochemist, as his name implies, inhabits a sort of no man's land between the classical fields of chemistry and biology. The field itself is not much more than a century old, since, until the 19th century, a theoretical barrier was supposed to exist between the inorganic world and the organic, or vital, world. The chemists who breached this wall and commenced to infiltrate the domain of biology were faced with formidable technical and theoretical difficulties and also, in many cases, with the antagonism of their biological colleagues. This antagonism is a natural consequence of different languages and different ways of thought. The biologist uses the language of semantics and is satisfied with a descriptive and qualitative theory. The chemist leans toward the language of mathematics and requires quantitative results. There is still another difference: chemists (and physicists) have great respect for the Reverend Occam's razor and endeavor to limit their assumptions to the minimal number essential for an explanation, in accordance with the principle of the conservation of hypotheses; whereas some biologists have no respect for the Reverend's weapon and fearlessly bolster an ailing (and unnecessary) assumption by another similar one. As a result, the chemist, who thinks he stands on firm ground, is frequently astonished to find himself facing a whole company of unnecessary assumptions, which he is expected to disprove, rather than lop off with William of Occam's weapon.

This incompatibility of temperament renders collaboration between chemists and biologists a difficult matter.

It is not surprising, therefore, that the history of biochemistry is a chronicle of a series of controversies, in several of which I have been more or less engaged. These controversies exhibit a common pattern. There is a complicated hypothesis, which usually entails an element of mystery and several unnecessary assumptions. This is opposed by a more simple explanation, which contains no unnecessary assumptions. The complicated one is always the popular one at first, but the simpler one, as a rule, eventually is found to be correct. This process frequently requires 10 to 20 years. The

¹ William of Occam (1270? to 1349?); "The Invincible Doctor." Occam's Razor: a method of argumentation adopted by William of Occam, consisting of eliminating all unnecessary facts or constituents from a question under analysis.—Ed.



John H. Hatting

PREFATORY CHAPTER

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reason for this long time lag was explained by Max Planck. He remarked that scientists never change their minds, but eventually they die.

The first and one of the most famous of these controversies centered about the synthesis of "organic" compounds, i.e., those derived from organisms. It was known that these compounds, when analyzed, were similar to "inorganic" compounds, but, since they came from living cells, it was generally considered impossible to synthesize them in the laboratory. Then Wohler synthesized urea, and the theoretical barrier between organic and inorganic chemistry was removed.

The next battles were fought over the nature of enzyme reactions. Spallanzani had shown in the eighteenth century that meat would digest outside the animal if it had been soaked in gastric juice. The hydrolysis of starch also had been caused to occur *in vitro*. Nevertheless, the more complicated reactions were still assumed to be "vital." The controversy centered about alcoholic fermentation, which Pasteur had shown occurred only when organisms were present. Here then was a typical vital process. Then Buchner put some yeast in a hydraulic press and caused fermentation with the cell-free extract.

The chemists thereby advanced into new territory, but were immediately met with the objection that the enzyme carried the mysterious vital principle and that enzyme reactions were qualitatively different from inorganic reactions.

Berzelius, early in the nineteenth century, had very shrewdly suggested that enzymes were a special class of catalysts, but this point of view was not generally accepted for more than 50 years—evidence of the great originality of the suggestion! Arrhenius, in his *Quantitative Laws in Biological Chemistry*, showed that all the various peculiarities of enzyme reactions could be found in inorganic reactions, and thus began the application of physical chemistry to vital processes [Arrhenius (1)]. He was violently attacked, especially for attempting to apply chemical theory to immunological reactions, but his viewpoint, of course, was correct and led to the elucidation of enzyme kinetics by Michaelis & Menten, and of immunological reactions by Landsteiner (6) and by Heidelberger. De Kruif and I showed that agglutination of bacteria was similar in every respect to that of other finely divided particles [Northrop & De Kruif (18)].

In the meantime, the chemical nature of the enzymes themselves remained in dispute. Their general properties were those of proteins, and about 1900 a protein was isolated from gastric juice by Pechelharng & Ringer, who considered it to be the enzyme pepsin. This conclusion was not accepted, and, as a result of repeated failure to isolate any enzyme, they were considered (by fallacious reasoning, this time by chemists) to be "neither fats, carbohydrates, nor proteins, but an entirely new and unknown class of compounds."

It was at this point that I became involved in the controversy. I had spent several years in the Department of Zoology at Columbia University,

where I had the good fortune to study under Morgan, Wilson, and Calkins, and to be associated with Muller, Altenberg, Bridges, and Sturtevant. I had then transferred to the Department of Chemistry (where I found Michael Heidelberger and George Scatchard). I worked with Nelson and Kendall and became fascinated by the enzyme puzzle. I then moved to Jacques Loeb's laboratory at the Rockefeller Institute as W. B. Cutting Traveling Fellow of Columbia University.

Loeb believed that the properties of living things could be completely explained in terms of physics and chemistry. In defense of his thesis he proved that the behavior of many plants and animals could be accurately predicted under certain conditions [Loeb (8)]. This theory of tropisms was attacked largely by anthropomorphic criticisms, none of which could have withstood the Reverend Occam's weapon. Tropisms, of course, are now a well-accepted part of experimental biology.

Loeb, by then, had planned and carried out his extraordinary experiments on artificial parthenogenesis, experiments that for originality of conception and brilliancy of performance have rarely been surpassed. They are still, I believe, the nearest approach to the creation of life [Loeb (7)].

In fact, if these experiments are interpreted in the same way that the "photo-reactivation" experiments have been interpreted, then artificial parthenogenesis does create life.

The failure to create life in the laboratory has given rise to a number of unnecessary assumptions which the Reverend Occam would never tolerate. It has been objected, for instance, that the probability of a living cell arising by chance is so small as to render the event impossible. But it must be remembered that what is a vanishingly small chance to us who work in terms of a few years may present no difficulty to natural forces which work in terms of millions of years.

The fact that optically active compounds are found only in living organisms also has been cited as evidence that living matter could not have arisen from non-living without the aid of some intangible force. There are several ways, however, by which optically active solutions may be prepared from optically inactive material without the introduction of living matter. The simplest method is random distribution of a racemic mixture of *d* and *l* crystals. Each individual crystal is optically active and will form an optically active solution [Northrop (14)].

To return to Jacques Loeb: At the time I entered the laboratory, he was about to attack the problem of the peculiar properties of proteins—in particular their osmotic pressure, viscosity, and swelling. Proteins at that time were considered to be "colloidal complexes" of no definite molecular weight, which combined with electrolytes by a process of adsorption. This explanation again contained unnecessary assumptions which could not survive the Reverend's weapon. Loeb was able, with the help of Donnan's theory of membrane equilibrium, to explain all the complicated phenomena of osmotic pressure, viscosity, and swelling [Loeb (8)]. This explanation rests on firmly

established chemical and physical theory. At the same time, Svedberg demonstrated that proteins have definite molecular weights, just like other substances [Svedberg (24)]. These results founded the modern theory of protein chemistry.

The solubility of proteins was another anomalous property that was supposed to distinguish them from other compounds. The solubility of most protein preparations increases as the quantity of solid phase increases. This may be interpreted in the usual two ways: (a) there is more than one component in the solid phase (Willard Gibbs's phase rule); or (b) proteins are not amenable to chemical theory. Kunitz and I were able to show that Gibbs's phase rule is correct [Northrop, Kunitz & Herriott (20)]. A pure protein has a definite constant solubility. Most protein preparations contain a group of similar proteins, and the solubility therefore increases with the quantity of solid. This is understandable since it is now known that animals produce slightly different proteins at different ages.

Loeb's interest and success with the chemistry of proteins led me to consider the protein nature of enzymes, and I repeated Pekelharing's & Ringer's experiments without being able to carry them further.

Then in 1926, Sumner described the isolation of a crystalline protein, which he considered to be the enzyme urease. This work solved the problem of the chemical nature of enzymes and so enormously increased the field of the chemist, since enzymes are uniquely characteristic of vital reactions. Sumner at that time had no convincing proof of the purity of his preparation, and very little attention was paid to his results for some years, since it was well known that enzymes were not proteins and so the protein must be a mere carrier.

Sumner's work encouraged me to repeat the pepsin experiments again, this time on a much larger scale. I finally succeeded in isolating and crystallizing a protein that is the enzyme pepsin. This protein is probably the same as that isolated by Ringer & Pekelharing 30 years before and buried under a series of unnecessary assumptions. The pepsin protein was also considered a "carrier" for years, and it required a long series of quantitative experiments by Kunitz, Herriott, Anson, and myself [Northrop, Kunitz & Herriott (20)] and by Sumner and his collaborators [Sumner & Somers (23)] before the simple explanation that the protein was the enzyme gained general acceptance.

The controversy over the chemical nature of enzymes, which had raged for over a century, was thus ended. In its place there appeared a very similar controversy over the chemical nature of viruses in general and bacterial viruses in particular.

The course of events, when a susceptible culture of bacteria (or a higher organism) is infected with a virus, is so similar, at least superficially, to the course of bacterial infection of a higher organism that most of the early workers took it for granted that the virus was a small parasite. This point of view was adequate at first, but the more the reaction was studied, the

more discrepancies appeared which required the introduction of more secondary assumptions. This was especially true of bacterial viruses.

It was found that some bacterial cultures produced virus continuously, without apparent harm, and that the spores of these cultures could be heated to boiling and still produce virus, although the virus itself was inactivated by much lower temperatures. The virus was found to be very small, much smaller than the smallest known organism; it has no metabolism and can not be grown apart from its "host." It is entirely resistant to some violent cell poisons, such as toluene and hydrocyanic acid; it may be inactivated (killed) and reactivated (resurrected) [Krueger & Baldwin (4, 5); Northrop (13); Heicken & Spicher (2)]. If the virus were alive, then these experiments are tantamount to the creation of life.

It is sometimes stated that bacteria and many other organisms that have been "inactivated" by ultraviolet light and then placed in the dark may be "reactivated" by visible light. There is no evidence, however, that the organisms are ever "inactivated"; this assumption is, therefore, unnecessary and may be lopped off with the Reverend Occam's weapon. It is sufficient to suppose that the ultraviolet light causes an injury which increases in the dark but decreases in the light or that a toxic product which is decomposed by visible light is formed by the ultraviolet light. If the organisms are left in the dark long enough to be sure they are "inactivated," they cannot be "re-activated."

The virus, on the other hand, may be inactivated and kept in that state for months, and probably years, and then reactivated in a few minutes.

A light-sensitive enzyme system, studied by Rupert, Goodgal & Herriott, also shows true inactivation and reactivation.

The fact that some viruses, at any rate, may be reactivated is of practical importance. It proves that negative tests for activity in a virus vaccine, for instance, are not conclusive, since the virus may, under certain conditions, become active again.

In order to maintain the idea of a living cell, the "obligative parasite" hypothesis was advanced. This assumption is, in fact, a whole series of assumptions: (a) the virus was originally a free-living organism; (b) it became a parasite; (c) it lost its metabolic processes; (d) it lost one of its nucleic acids but kept the other; (e) it gained the power to resist many cell poisons; (f) it gained the power to come to life after being killed; and (g) there is a vital force in living cells that cannot be replaced by any experimental procedure or condition. This last assumption is implicit in the term "obligative parasite." If this term is taken to mean that the parasite has not been cultivated apart from its host, then the term is merely a statement of the facts, in different form. If it is taken to mean that it is theoretically impossible to cultivate the parasite apart from its host, then this is tantamount to the assumption of a vital force.

An equally logical (and unnecessary) hypothesis would assume that a virus is an early stage in the development of a free-living parasite.

If a virus is to be classified as a living organism, it is necessary to have a definition of living, and this is not an easy thing to do. In fact, Pirie, a brilliant researcher in this field, considers it to be impossible. The criterion of self-reproduction will not suffice, since crystals reproduce and enzymes also.

Living organisms were supposed to be unpredictable in contrast to non-living, but this distinction is not valid either. The physicists tell us that nuclear particles are also unpredictable, so we may conclude that (a) such particles are alive, or (b) non-predictability (i.e., free will) is not restricted to the living.

I have defined a living system as one that can use energy to carry out the synthesis of more of itself. The concept of the use of energy rules out all known autocatalytic reactions and also the growth of crystals, although they reproduce themselves, since these events occur with the liberation of energy. That is to say, they run downhill, while a living organism climbs uphill [Northrop (11)].

If this definition is accepted, a virus is not alive, since it has no metabolism; neither is an enzyme, for the same reason, although some enzymes may be propagated.

The polymerase system, discovered by Kornberg (26) and his collaborators, is able to synthesize DNA by an autocatalytic reaction that requires energy. It is possible that this reaction represents the simplest living system.

Bordet pointed out that the properties of a virus could be explained more simply by the assumption that, like an enzyme, it is a product of the metabolism of the host cell. Gratia noted the similarity between a virus and the "transforming principle," which controls the type of some bacterial cultures. This suggestion was not much help at the time, since the nature of the transforming principle was as little known as that of the virus. In view of future developments, however, it was a very shrewd guess.

It has been suggested repeatedly that the virus is a gene, or related to a gene, but, since no one knows what a gene is or even if such a thing exists, this suggestion is not much help either.

The controversy thus assumed the usual character: a popular hypothesis containing complicated and unnecessary assumptions and a simple and unpopular hypothesis.

This question of the nature of the virus is of great practical, as well as theoretical, interest to those who work with them, since, if a virus is alive, the plan of research would be quite different from that planned if it is the product of the metabolism of the host. For instance, if the virus is alive, attempts to isolate it would follow the technique used in the culture of organisms, whereas, if it is similar to an enzyme, the methods used in isolating enzymes would be chosen. All attempts to isolate a virus by cultural techniques have failed, while attempts to isolate them by enzyme (protein) techniques were successful, or nearly so, almost at once. Stanley isolated and crystallized a nucleoprotein which he thought was tobacco mosaic virus;

Bawden & Pirie crystallized one which they thought was bushy stunt virus; and I isolated, but did not crystallize, one which I thought was a bacterial virus [Northrop (10)]. Many other bacterial and plant viruses have since been isolated by the same general technique and crystallized. All are nucleoproteins, containing either ribose or deoxyribose nucleic acid, but not both.

These preparations were accused at first of containing some cells, but no one could find any cells, and it became generally accepted that the nucleoprotein represented the virus "itself."

There were, however, some facts that did not agree with this conclusion. Some bacteria, which are capable of producing virus, do not contain any virus nor even any virus antigen. This fact rules out any explanation for the growth of the virus (if it is a nucleoprotein) which entails either cell-division or autocatalysis. This difficulty led me to suggest [Northrop (12)] that the nucleic acid alone was the essential part of the virus. This guess was soon corroborated by Hershey & Chase in the case of bacterial virus, and by Gierer & Schramm, Fraenkel-Conrat, Cheng, and others in the case of other viruses.

This final step in the isolation of the virus, the removal of the protein, consists in extracting a solution with phenol, an operation which is rather unorthodox as a means of isolating a parasite.

The viruses are, therefore, closely related in chemical structure to the transforming principle, which Avery, MacLeod, & McCarty had shown to be a nucleic acid also.

The biological properties of the virus are also related to those of the transforming principle, since a virus may transfer the same type of genetic information as does the transforming principle. Freeman found, for instance, that virus from a virulent diphtheria culture would transform an avirulent strain of the organism to a virulent one. This property of the virus to transfer genetic information was shown by Lederberg to be of very general occurrence. He named it "transduction" and pointed out that it was very similar to transformation. A number of cases have been described in which enzyme formation is transmitted by a virus.

The discoveries of the nucleic acid nature of the transforming principle and of the viruses are of the greatest importance, since they identify the chemical nature of the master molecule which determines the hereditary character of the cell. As a result, the chemist is now able to attack the problem of the structure of the gene and so determine the chemical basis of heredity. In fact, it already appears probable that a gene is a molecule of nucleic acid, and a mutation is a change in the structure of this molecule [Mundry & Gierer (9)].

If the virus is related to the transforming principle, it probably is formed in the same way, i.e., as a result of a mutation of the "host" cell. As a result, the cell produces virus particles instead of another cell [Northrop (17)].

This hypothesis makes unnecessary any assumption in regard to the

origin of the virus as an independent organism or to the presence and properties of provirus, or virus anlage, other than the conventional genes, in the cells.

Somewhat similar ideas have been advanced by Dugger & Armstrong, Muller, Bordet, Wollman, and Darlington, and recently by Bawden, by Romanzi, Cavallero & D'Adda, and by Euler.

It might be supposed that the proposed mechanism for the production of a virus predicts that mutagenic agents should cause the appearance of a virus in any population. This is not the case. Experience has shown that mutagenic agents do not create new mutations, but only increase the incidence of those already present. Further, the increase is rarely more than 100-fold. It follows that a "new" virus can only be expected to appear as a result of treatment with a mutagenic agent (in the case of bacterial virus) if the culture already contains one mutant per 10^{10} to 10^{12} cells.

If it contains more than that proportion, the virus would be detected without treatment; this is the usual condition. If it contains less, the virus could not be detected even though the mutagenic agent increased its incidence 100-fold. The chance of finding a culture that produces just the proper number of virus-producing mutants is, therefore, very small. A further difficulty is due to the fact that it may be necessary to discover an indicator strain in order to detect the virus after it has been produced.

The appearance of a virus in a non-infected and non-lysogenic bacterial culture has been reported by Herčík (3), by Romanzi, Cavallero & D'Adda (21), and by Rutberg & Hedén (22). In these experiments, the new virus attacks the cultures from which it arose, and the result, therefore, cannot be ascribed to induction of a "lysogenic" culture since the viruses derived from such cultures do not attack the bacteria that produce them. The production of silkworm disease virus as a result of treatment of silkworms with various mutagenic substances has been repeatedly reported by Yamafuji (25).

These facts, of course, may be explained on the basis of an obligative parasite, but, in order to do so, it is necessary to invoke a number of secondary assumptions, none of which would survive the Reverend's razor. For instance, if a virus, i.e., a nucleic acid, is considered to be a parasite that has lost its other parts, then enzymes, proteins, carbohydrates, and fats are parts of a parasite which were lost. These compounds have retained their parasitical nature, however, since, with the exception of some enzymes, they cannot propagate *in vitro*, but only in the "host" cell. Also transforming principles must be remains of parasites, since their biological and chemical properties are the same as those of viruses.

From the present point of view, a normal culture of bacteria consists principally of virus-free, virus-resistant, and (for example) penicillin-sensitive cells. Occasionally a cell will appear which produces a new nucleic acid (virus particle) and this nucleic acid may transmit the property of producing more of itself to another "sensitive" cell. In the same way, an occasional cell may appear which is resistant to penicillin; this cell will produce a new nucleic

acid (transforming principle), and this nucleic acid may transmit the property of penicillin resistance to other "sensitive" cells.

The main distinction between a virus and one of the transforming principles then lies in the fact that the virus, in general, transfers a harmful property, while the transforming principle, in general, transfers a useful property. The production of a virus, therefore, is similar to most mutations, since mutations, in general, are harmful.

Both virus-producing cells and penicillin-resistant cells may occasionally revert to the original virus-free, virus-resistant, penicillin-sensitive cell as in the original culture.

This assumption concerning the origin of a virus may be tested experimentally in several ways. It is known, for instance, that mutagenic agents increase the number of all mutants present in a population to about the same extent.

Lwoff and his collaborators have shown that mutagenic agents, in general, increase the number of virus-producing cells in bacterial cultures. If this effect is a mutation, then there should be a corresponding increase in the number of other known mutants present in the culture.

In order to test this prediction I have determined the effect of ultraviolet light, hydrogen peroxide, and heat on virus-producing cells and terramycin-resistant mutants and of manganese chloride on streptomycin-resistant mutants and virus-resistant mutants, in addition [Northrop (15, 16)]. All the different types increase to about the same extent in the presence of the mutagens. Also the rate of production of the virus and the equilibrium ratio of virus to cells is predictable by equations similar to those that Kunitz and I found to predict the proportion of the terramycin-resistant mutants in bacterial cultures [Northrop & Kunitz (19)].

At the present time, therefore, the virus controversy is in the same condition as was the enzyme controversy 50 years ago. There is a simple and unpopular hypothesis and a complicated and popular one, and another 30 years may be needed to reach a decision. What this will be cannot be predicted, but what can be predicted is that, when this controversy is ended, another similar one will take its place.

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BIOLOGICAL OXIDATION^{1,2}

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In the period covered by this review, the relationship of structure to mechanism of action has been of primary interest to biochemists. This relationship has been investigated at both the molecular and cellular level. In oxidative phosphorylation, the relationship of the cellular particulate to chemical mechanisms has been established to be of particular importance. A majority of the investigations in this area have employed material derived from mammalian sources, and, therefore, investigations which bear directly on biological oxidation in mammalian systems have been emphasized.

A number of recent books have reviewed aspects of biological oxidation (1 to 5), and several excellent symposia have become available (6 to 10). Reference will be made to both sources in this article.

CYTOCHROME-A

Cytochromes-*a* and -*a*₃, the terminal respiratory enzymes located in the mitochondrial matrix, are difficult to extract and to prepare free from other hemoproteins, extraneous protein, and lipid. Current efforts to obtain more highly purified preparations of these electron-transport components have turned to various procedures for their solubilization. Yonetani (11) has studied the effects of detergents in solubilizing and maintaining the enzyme activity of the cytochrome-*c* oxidase. Igo *et al.* (12) made a preparation of cytochrome oxidase by fractionation of the NADH₂⁵ oxidase preparation;

¹ The survey of the literature pertaining to this review covers the period from September, 1959, to October, 1960.

² The following abbreviations are used: FAD for flavin-adenine dinucleotide; NAD for nicotinamide-adenine dinucleotide; NADase for nicotinamide-adenine dinucleotidase; NADH₂ for nicotinamide-adenine dinucleotide, reduced form; NADP for nicotinamide-adenine dinucleotide phosphate; NADPH₂ for nicotinamide-adenine dinucleotide phosphate, reduced form.

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⁴ The author wishes to acknowledge the helpful suggestions of Dr. Elmer Stotz, Dr. Britton Chance, Dr. Ronald Estabrook, and Dr. Emanuel Margoliash.

⁵ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH₂), for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH₂), for triphosphopyridine nucleotide, reduced form (TPNH).

this preparation lost up to 90 per cent of its activity when suspended in 5 per cent sucrose, but the activity could be restored by use of such surface-active agents as deoxycholate or lysolecithin.

Gibson *et al.* (13) studied a preparation obtained by partial hydrolysis with venom and fractionation on calcium phosphate gel. Criddle & Bock (14) studied a preparation of soluble cytochrome oxidase in the ultracentrifuge and found that it was highly polymerized.

Based on the original suggestion of Keilin & Hartree (15) that cytochrome-*c* oxidase contains copper, positive correlations have been made between copper in the diet and cytochrome oxidase activity. Howell & Davison (16) studied the copper content of the tissues of sway-back lambs, a disorder associated with copper deficiency. In general, the differences in cytochrome oxidase activity between the sway-back and normal animals could be correlated with similar alterations in the copper content of the respective organs. The cytochrome oxidase activity and the copper content in the brain of these animals were decreased as compared to normal, although the succinic dehydrogenase activity was normal, indicating that a decrease in enzyme concentration is not general.

Recent studies from three different laboratories on the copper content of the cytochrome-*c* oxidase preparations tend to corroborate the view that copper is an important structural component of cytochrome-*c* oxidase. Takemori *et al.* (17, 18) found that their purified preparation of "cytochrome-*a*" contained one atom of copper per mole of cytochrome-*a*. They further noted that the copper was present at all stages of purification in at least this ratio. Wainio *et al.* (19) also found a positive correlation between the copper content, the heme content, and cytochrome-*c* oxidase activity, and Mackler & Penn's (20) analyses indicate a positive correlation between copper and heme content.

Takemori (18) and Vander Wende & Wainio (21, 22) are not, however, in complete agreement on the state of copper in cytochrome oxidase preparations. Takemori *et al.* (17, 18) indicated that the copper in cytochrome oxidase is not released by dialysis against copper-chelating agents, although there is loss of activity when they are added. On the other hand, Wainio and his co-workers (21, 22) have found that the copper in cytochrome-*c* oxidase can be removed by dialysis against a number of copper-chelating agents with a resulting decrease in activity, and, as the copper content decreased, the cytochrome-*c* oxidase activity of the preparation decreased. Takemori found that 50 per cent of the cytochrome-*c* oxidase activity was inhibited by copper-chelating agents, whereas the copper in the cytochrome-*c* oxidase preparation was not released by dialysis against these reagents. They did find, however, that acid treatment of the cytochrome-*c* oxidase released the copper and that the copper in the cytochrome-*c* oxidase preparation appeared to be in the divalent state. Although they could show that cuprous copper was present, the authors felt that this was because the —SH content of the preparations reduced the copper and that the copper actually existed in the preparation in the cupric form.

Vander Wende & Wainio (21), in contrast, through the use of 2,2'-di-quinoline, a reagent specific for cuprous ion, and cuprizone, specific for cupric ion, found that approximately 85 per cent of the copper in an oxidized enzyme preparation was in the cuprous state. They suggested that the copper may not participate in electron transport.

Sands & Beinert (23), however, employing paramagnetic resonance, obtained a signal when the cytochrome-*c* oxidase preparation was in the oxidized form; no signal was given when the preparation was in the reduced form. The rate and extent of the signal was a function of the age of the preparation, its deoxycholate concentration, the concentration of the reductant, and the oxygen present. Added cupric ions did not affect the signal, which led these investigators to believe that the copper in the cytochrome oxidase may participate in electron transport.

The question of the individualities of cytochromes-*a* and -*a*₃ is one of the major areas in which there is no uniform agreement. On the basis of the spectrum of cyanide and carbon monoxide compounds with yeast and heart muscle preparations, Keilin & Hartree (24) had concluded that two cytochromes, which they labeled cytochromes-*a* and -*a*₃, have absorption peaks in the same region of the spectrum. Subsequent work in many laboratories (25 to 30), using various preparations, has apparently confirmed this point of view.

Yonetani (31) has attempted to determine quantitatively what parts of the absorption of the two peaks can be attributed, respectively, to cytochromes-*a* and -*a*₃. On the assumption that cytochrome-*a* does not react with cyanide or carbon monoxide whereas reduced cytochrome-*a*₃ will react with both, he initially determined the absolute and difference spectra of purified preparations of cytochrome-*c* oxidase. The reduced cyanide compound undergoes auto-oxidation, whereas the oxidized cytochrome *a*₃-cyanide compound is not easily reduced. Proceeding on these assumptions, Yonetani was able to determine that reduced cytochrome-*a*₃ accounted for over 50 per cent of the absorption in the region of the Soret peak but only 28 per cent of the absorption at the 605 mμ peak. Thus, the ratio of the extinction of the Soret peak to the alpha peak in the visible region is 4.5 for the reduced form of cytochrome-*a* and 13 for the reduced form of cytochrome-*a*₃.

The view that the terminal respiratory enzyme is made up of two components is not universal. Okunuki *et al.* (32) and Wainio (22) have supported the position that cytochrome-*c* oxidase is a single hemoprotein, cytochrome-*a*. The former group has further indicated that this cytochrome-*a* in the reduced form will produce a complex with oxygen which has absorption maxima at 426 to 428 mμ and 603 mμ. This cytochrome-*a* oxygen complex is not auto-oxidizable in the absence of cytochrome-*c* but auto-oxidizes quickly on the addition of small amounts of cytochrome-*c*.

Minnaert (33) has also demonstrated spectroscopically that a complex of oxygen and the reduced cytochrome-*c* oxidase occurs. The spectral properties claimed for this oxygen complex are, however, different from those reported by the Japanese workers. It is interesting to note that kinetic studies (34)

which employed spectrophotometric and magnetometric measurements failed to detect an oxygen complex in the copper-containing oxidase, laccase.

Wainio & Greenlees (35) have investigated cytochrome-*c* oxidase in particulate heart muscle preparations. They determined that there are two binding sites for cyanide on cytochrome-*c* oxidase, whereas carbon monoxide has a single binding site. The amount of cyanide bound will vary, depending upon the presence of reduced cytochrome-*c*. It was suggested that copper might be involved in the cyanide-sensitive sites.

In other investigations of the interaction of cytochrome-*c* and cytochrome-*c* oxidase, Smith & Conrad (36) have found that cytochrome-*c* itself, as well as other basic proteins, such as salamine, inhibited the cytochrome-*c* oxidase activity of particulate and solubilized preparations. Similar observations have been made on the cytochrome-*c* peroxidase system (37). Estabrook (38), studying the influence of cytochrome-*c* in restoring the succinic oxidase activity of a particulate heart muscle preparation that was deficient in cytochrome-*c*, showed that inorganic cations, but not anions, compete reversibly with cytochrome-*c*. Presumably, the competition was for a specific locus on the particles. Trivalent cations were the most effective competitors and monovalent cations were the least.

The effect of concentration of ions and pH on the cytochrome-*c*-cytochrome-*c* oxidase reaction has also been studied (39). It was found that the molarity of the buffer had a pronounced effect. The pH optimum of the reaction shifted to lower values as the molarity of the buffer was increased up to 0.1 *M*, and at higher concentrations the pH optimum was constant. There was an increase in enzyme activity with increased concentrations of the buffer up to the same molarity, whereas the activity decreased at higher concentrations. The effects observed could be related to the ionic strength or cation concentration, since monovalent cations were employed and optimum activity in terms of ionic strength was found to be independent of the pH. The authors (38, 39) agreed that the effects of cations are a function of the charge of the cation.

One of the major puzzles in oxidative metabolism is the mechanism by which molecular oxygen oxidizes the terminal respiratory enzyme, cytochrome-*c* oxidase. It is difficult to reconcile the fact that an apparent one-electron transport system, such as the cytochromes, is able to reduce oxygen, which requires four electrons, without the appearance of detectable intermediates.

Wang & Brinigar (40) have attempted to clarify this question by designing a synthetic catalyst for the oxidation of cytochrome-*c*. They prepared a type of polymer by reacting hemin, a bidentate ligand, and a poly-cation in the proper order. Three different types of catalysts were made with 4,4'-dipyridyl, 1,2-di-4-pyridylethylene, and 1,2-di-4-pyridylethane, respectively, as the connecting ligands. The various polymers were then tested for their ability to catalyze the oxidation of reduced preparations of cytochrome-*c*. The results showed that the ligand with the conjugated double bond system,

which linked the different heme groups, played an important part in the catalysis. The transfer of the electrons between the different heme units was greatly facilitated by the use of ligands connected by the conjugated double-bond system. From this, the authors inferred "that the oxygen molecule which was linked to the terminal heme group was reduced directly to water through a four-electron transfer mechanism."

Lundegardh (41) studied the temperature coefficient of the terminal respiratory unit between 5° and 35–40° and determined that, insofar as the reduction is concerned, the system follows the usual course for chemical reactions, with Q_{10} of two until 40–45°, at which temperature some denaturation of proteins takes place. The velocity of the reoxidation is a reversed image of that for reduction. The highest velocity is attained at 5°, and a minimum is reached at 40–45°. In the over-all cytochrome-*c* oxidation, an increase in temperature produces a net increase in the velocity of the reaction, but with a lower Q_{10} than that observed for most enzymatic reactions.

The prosthetic group of cytochrome oxidase, which is the iron porphyrin nucleus of cytochromes-*a* and -*a*₃, has been studied extensively. Hemin-*a* is probably the prosthetic group of both cytochromes-*a* and -*a*₃ (42, 43). The definitive work of Marks *et al.* (44) has established the nature of the deuteroporphyrin derived from hemin-*a*, and Piatelli (45) has determined the position of the formyl group. Morrison *et al.* (46) have suggested a possible structure for hemin-*a* which varies from that proposed by Lemberg *et al.* (47).

Soluble cytochrome oxidase preparations undergo spectral changes on standing at 4°. In general, spectral changes result in a decreased absorption in the visible region, as well as in the Soret region, with the position of the Soret peak shifting to lower wavelengths. These spectral changes are accompanied by decreased enzymatic activity.

The change in the spectrum is apparently the result of changes which take place on the prosthetic group of the cytochrome. Two hemins, hemin-*a* and cryptohemin-*a*, can be isolated from the preparations of cytochrome-*c* oxidase (48, 49). There appears to be destruction of the hemin during the aging, and the ratio of cryptohemin-*a* to hemin-*a* increases with increased time of aging. Elliott *et al.* (49) have not found cryptohemin-*a*, but an entirely different hemin in their aged preparation. This group claims that aging of cytochrome-*c* oxidase preparations results in a mitochondria-like pigment. An identical absorption spectrum of mitochondria-like material is also obtained by the aging of cytochrome-*b* preparations, although the hemin groups of the cytochrome-*b* and cytochrome-*c* oxidase and the hemins resulting from the aging are quite different.

CYTOCHROME-C

As work continued on the interrelationship of structure and function of cytochrome-*c*, improved procedures for its isolation were developed. These clearly demonstrated that, despite its stability, the cytochrome-*c* structure is affected by isolation methods. Detailed work on the primary structure of the

amino acid sequence of cytochrome-*c* has also continued and is rapidly approaching the point where the total cytochrome-*c* structure will be clarified. A detailed account of this work will be found in this volume in the section on hemoproteins by Margoliash (50).

CYTOCHROME-*B₅*

Estabrook (51) has employed low temperature spectroscopy to investigate the cytochromes and has reviewed the method of recording the spectra of the hemoproteins at the temperature of liquid N_2 . These procedures resulted in a five- to tenfold intensification of absorption bands. It is interesting to note that, in the case of cytochrome-*b₅*, the splitting of the α bands has indicated the possibility that preparations of *b₅* may contain more than one pigment (51, 342).

Strittmatter reviewed the work which led to the conclusion that cytochrome *b₅* was localized in the microsomes and indicated its characteristics and possible biological significance, but Raw and co-workers (53) have substantiated their position that *b₅* is present in liver mitochondria. Nucleic acid determinations ruled out the possibility that their preparation of mitochondria was grossly contaminated with microsomal material. Contamination of mitochondria up to 15 per cent by microsomes could not account for the amount of *b₅* present.

Raw & Colli (54) have prepared hexagonal crystals of cytochrome-*b₅* from pig liver homogenates. These were reducible by $NADH_2$ -cytochrome-*b₅* reductase. Strittmatter (55) isolated large quantities of cytochrome-*b₅* from calf liver microsomes. The preparation was homogeneous in the ultracentrifuge and on electrophoresis in phosphate buffer at pH 7.2. The molecular weight, based on heme content, was 12,700, which agrees with sedimentation determinations.

With this preparation, the author attempted to determine the nature of the groups involved in binding the heme prosthetic group to the apoenzyme. The prosthetic group was removed by treatment with acidified acetone, and the ability of the apoenzyme to recombine with the heme after treatment with various reagents was investigated. The recombination of the protohemin with apoprotein is not inhibited by *p*-chloromercuribenzoate, demonstrating that sulfhydryl groups are not involved in heme binding. Tyrosyl residues are likewise not involved, since the recombination of prosthetic group and protein took place after iodination. However, acetylation of the protein indicated that an amino group apparently is involved in heme binding. The participation of an imidazole group is indicated by the lack of recombination when the iodinated and acetylated protein was treated with diazotized acid. This bears out an earlier theoretical consideration (56).

When Krisch & Staundinger (57) investigated the role of cytochrome-*b₅* in adrenal microsomes, they found that the microsomes did not oxidize $NADH_2$ at any appreciable rate. However, when ascorbic acid is added to the system, the pyridine nucleotide is oxidized, and the addition of purified cyto-

chrome- b_5 enhances the oxygen consumption (58). This complete system is not sensitive to any of the common inhibitors, such as cyanide, antimycin-A, dinitrophenol, dicumarol, or amytal. The authors found that cytochrome- b_5 catalyzes the oxidation of ascorbate but that in the presence of NADH_2 and microsomes the ascorbate remains reduced. They postulate that the oxidized cytochrome- b_5 reacts with ascorbate, producing a monodehydroascorbate which is, in turn, reduced by NADH_2 .

CYTOCHROME- B_2

Cytochrome- b_2 was isolated from yeast as a crystalline protein containing a deoxyribose polynucleotide component (59 to 61). The polynucleotide (62) could be separated by a number of methods, leaving a protein which could not be crystallized but which retained full enzymatic activity. The molecular weight of the crystalline cytochrome- b_2 was estimated to be 160,000, and that of the nucleotide-free enzyme was 150,000. By spectral and chemical analyses, the cytochrome preparation was shown to contain protohemin and riboflavin-5'-phosphate in equimolar concentrations and one hemin group per 80,000 molecular weight. The authors suggest that the crystalline material, which they identify as a flavohemoprotein, is a dimer.

On the other hand, Yamashita *et al.* (63) had earlier extracted and crystallized a pigment from bakers' yeast which was labelled cytochrome- b_2 since it had the appropriate spectral properties. This preparation contained one hemin group per mol. wt. of 22,000. It had no flavin or non-hemin iron, and demonstrated no lactic dehydrogenase activity in either the presence or absence of flavin.

Both groups (61, 63) studied the question of the interrelationship of cytochrome- b_2 and the over-all lactic dehydrogenase system. They investigated whether there were two distinct proteins, one a flavoprotein dehydrogenase and the other a hemoprotein which acts as the specific electron acceptor for the dehydrogenase. Morton and co-workers (61, 62) concluded that the enzyme was a single flavohemoprotein; they based their opinion on the fact that the protein to hemin ratio remained constant in an experiment in which the DNA was separated by electrophoresis at pH 8.8. They further noted that, regardless of the procedure by which the enzyme was obtained, this ratio remained constant.

The cytochrome- b_2 of Yamashita and co-workers (63, 64) can be reduced enzymatically by preparations of yeast lactic dehydrogenase. However, under anaerobic conditions at pH 6.4, cytochrome- c is reduced 42 times as fast as cytochrome- b_2 . It was also noted that the pH activity curves for cytochrome- c and - b_2 reductions were quite different.

Lactic dehydrogenase, which contains both the flavin and hemin prosthetic group, has been described as auto-oxidizable. Lactate, however, inhibits the reaction with oxygen (65) and with the purified enzyme under optimal conditions, oxygen is a poor electron acceptor as compared to ferricyanide. The fact that catalase does not affect the reaction with oxygen and that the

flavin-free crystalline cytochrome- b_2 is auto-oxidizable has implicated the hemin as the site of the reaction with oxygen. It has been suggested, therefore, that the flavin moiety greatly modifies the reactivity with oxygen in the lactic dehydrogenase which contains both prosthetic groups.

Hasegawa & Ogura (66) investigated the relationship of the flavin moiety and the cytochrome or heme moiety and found that under anaerobic conditions both were reduced. Under these conditions, methylene blue will oxidize the flavin group whereas the cytochrome moiety remains in the reduced form. They were also able to replace the riboflavin-5'-phosphate prosthetic group of the enzyme with riboflavin. The riboflavin enzyme complex was enzymatically inactive.

These data enabled the authors to confirm two points: the hemin to flavin ratio is 1:1, and the probable sequence of transfer is from the substrate directly to the flavin mononucleotide of the enzyme, thence to the hemin moiety.

Nygaard (67 to 70) has separated several different forms of the yeast lactic dehydrogenase. Three forms have been separated on diethylaminoethyl cellulose columns and contain fractions with different absorption spectra and enzymatic properties as well as different flavin-hemin ratios. Two of these forms reduce cytochrome- c , ferricyanide, or 2,6-dichlorophenolindophenol at about the same rate, whereas the third form reduced the dyes very slowly. The former pair reduced b_2 , but decomposed on standing, yielding active enzymes which by-passed b_2 in the reduction of cytochrome- c .

CYTOCHROME-B

New preparations of cytochrome- b , free from cytochrome- c_1 , have been reported (71 to 73). In the preparation of Doeg and co-workers (72), 60 to 85 per cent of the cytochrome is reducible by succinate. The particle will also reduce ubiquinone when mitochondrial lipid extract is added. The cytochrome- b is apparently not required for the reduction of coenzyme Q (74).

Slater & Colpa-Boonstra (75) have studied the kinetics of the oxidation and reduction of cytochromes of heart muscle preparations with special emphasis on cytochrome- b . In agreement with the earlier work of Chance (76, 77), it was found that, in the presence of cyanide, cytochrome- b was reduced more slowly than a component participating in the main pathway of oxidation. Slater, however, interprets this slow rate as being characteristic of a succinate cytochrome- b reductase system which is inhibited by cyanide, and he suggests that cytochrome- b is on the main pathway of succinate oxidation. Keilin & King (78) recently studied the effect of cyanide and concluded that the cyanide reacts slowly and irreversibly with succinic dehydrogenase itself. This cyanide effect can, however, be prevented by succinate or dithionite and is, therefore, of little importance except in experiments which involve prolonged incubation in its presence.

Although evidence suggests that 2,3-dimercaptopropanol (BAL) and antimycin act at different sites, the position of the antimycin site is not clear.

TABLE I
RECENTLY DETERMINED VALUES FOR CYTOCHROMES

Cytochromes	Spectral Data	Potential
a^*	Ratio, 445 $m\mu$ /605 $m\mu$ (reduced) = 4.5/1 (31)	
a_3	Ratio, 444 $m\mu$ /605 $m\mu$ (reduced) = 13/1 (31)	
CN complex	Ratio, 444 $m\mu$ /605 $m\mu$ (reduced) = 6.2/1 (31)	
CO complex	Ratio, 430 $m\mu$ /590 $m\mu$ (reduced) = 7/1 (31)	
b	$E = 102 \text{ gm.}^{-1} \text{ cm.}^2$ (71) 561 to 575 $m\mu$ reduced	77 mv. (86)
b_2	$E = 38.8 \text{ mM}^{-1} \text{ cm.}^{-1}$ (60) 556.5 $m\mu$ reduced	12 mv. (66)
b_5	$E = 114 \text{ mM}^{-1} \text{ cm.}^{-1}$ (55) 413 $m\mu$ oxidized	
c	$E = 8.9 \text{ mM}^{-1} \text{ cm.}^{-1}$ (87) 550 $m\mu$ oxidized $29.9 \text{ mM}^{-1} \text{ cm.}^{-1}$ (87) 550 $m\mu$ reduced	245 mv. (88) 280 mv. (337)
c_1^\dagger	$E = 24.1 \text{ mM}^{-1} \text{ cm.}^{-1}$ (84) 15.3 $\text{mM}^{-1} \text{ cm.}^{-1}$ (83) 553 or 554 $m\mu$ reduced $E = 17.1 \text{ mM}^{-1} \text{ cm.}^{-1}$ (84) 9.4 $\text{mM}^{-1} \text{ cm.}^{-1}$ (83) 553 or 554 $m\mu$ reduced—oxidized $E = 116 \text{ mM}^{-1} \text{ cm.}^{-1}$ (84) 91.34 $\text{mM}^{-1} \text{ cm.}^{-1}$ (83) 418 $m\mu$ reduced	220 mv. (88)

* Prosthetic group hemin- a (42, 46, 47); mol. wt. = 880; Cu/hemin ratio = 1 (17, 18, 22).

† Prosthetic group mesohemin derivative linked by thioether to protein (83).

There is agreement that antimycin does not inhibit the reduction of ubiquinone but does inhibit its oxidation (79, 80). From other work (81), it has been concluded that the antimycin inhibits the respiratory chain between cytochrome- b and the BAL-sensitive factor. Repeating the work of Chance

(340, 341), Slater & Colpa-Boonstra (75) have suggested that antimycin reacts directly with the cytochrome-*b*. This was proposed to explain why the addition of antimycin to a succinate-reduced heart muscle preparation results in an increased intensity and a slight shift in the Soret peak of cytochrome-*b*. According to this concept, the increased absorption is caused by the reduction of another form, "cytochrome *b'*," which is reduced because the antimycin complex has an increased oxidation-reduction potential.

CYTOCHROME-*c*₁

Procedures for the purification of cytochrome-*c*₁ have been presented (82 to 84). The prosthetic group is closely related, if not identical, to that of cytochrome-*c* and can be removed by silver sulfate cleavage (83, 85). The reduction of cytochrome-*c*₁ preparations by succinate cytochrome-*c*₁ reductase preparations is antimycin-sensitive. The reaction of *c*₁ with "cytochrome-*a*" preparations is very slow. However, on the addition of small amounts of cytochrome-*c*, the *c*₁ is rapidly oxidized. Thus, cytochrome-*c* mediates the transfer of electrons between *c*₁ and "cytochrome-*a*."

Although detailed kinetic studies of the various cytochrome components in mitochondria have been described (10), no information has been obtained on cytochrome-*c*₁, since its absorption is obscured by cytochrome-*c*. At low temperatures, however, *c*₁ can be distinguished from *c* in preparations containing both components (51). In experiments designed to take advantage of this fact, Chance (77) has demonstrated that *c*₁ is, in all probability, a fully active member of the respiratory chain.

LIPIDS

The role of lipids and quinones in biological oxidation is under constant investigation, and conflicting data continue to appear. These materials are credited with two possible roles, one structural and the other functional.

In the first role, the lipids merely supply the appropriate environment for the sequence of oxidation-reduction reactions, whereas, in the second role, the lipids participate, are intermediaries in the electron-transport chain, and are oxidized and reduced or involved less directly in the phosphorylative mechanism.

The role of lipids in biological oxidation has been investigated by nutritional studies of vitamin E-deficient animals. In growing rats, lack of the vitamin appears to have varied effects on different tissues (89). A comparison of oxygen consumption in homogenates of control and deficient animals showed no difference in heart and liver when succinate was used as a substrate. However, there was a decrease in the oxidation in the kidney and brain of the deficient animals, whereas testes consumed twice as much oxygen.

Rats fed selenium and a vitamin E-deficient diet developed a fatal necrotic liver degeneration. Studies of oxygen consumption of liver slices from these deficient animals showed a decreased respiratory rate when glucose was

used as a substrate (90). This decline continued until the death of the animal. When tocopherol was injected into the deficient animals, however, the respiratory decline ceased. The ability of the various tocopherols, tococls and derivatives to reverse the decline in respiration in liver slices was investigated, and a marked difference in their efficiency was noted. Even compounds that were most effective when injected were without effect on respiration when added *in vitro* to the tissue slices. However, 2(3 hydroxy-3 methyl-5 carboxyl) pentyl-3,5,6-trimethylbenzoquinone and its γ -lactone, which are metabolic products of tocopherol isolated from the urine of rabbits and humans, prevented respiratory decline *in vitro* and even stimulated oxygen consumption.

The possibility that vitamin E plays a role in maintaining the structural integrity of cellular components, primarily because of its antioxidant properties, has also been considered (91, 92). The extent of peroxidation of lipids in various tissues was estimated by determining the amount of peroxide formed when tissue homogenates were incubated (93). The amount of lipids peroxidized by such procedures was a function of vitamin E in the diet. In animals fed a vitamin E-depleted diet, all the tissues rapidly formed peroxides; however, when the diet contained adequate vitamin E, only the brain tissues formed peroxides. Tissues of the vitamin E-deficient rabbits showed that lipid peroxidation took place even *in vivo* (94), demonstrating that vitamin E can function against oxidative degradation and thus aid in maintaining structure and function of cellular components.

The dehydrogenases were implicated when Rosenkrantz & Laferte (95) found that, with isocitric or α -ketoglutaric acid as a substrate, there was a decreased ability to reduce tetrazolium salts in skeletal muscles of vitamin E-deficient rabbits. However, Allen *et al.* (96), also employing vitamin E-deficient rabbits, noted that the muscles of animals in terminal stages of deficiency have an elevated cytochrome-*c* reductase activity per mg. of tissue, although cytochrome-*c* oxidase activity was normal. Corwin & Schwartz (97) made similar observations with liver mitochondria obtained from deficient rats. When no vitamin was present in the tissue of vitamin E-deficient chicks, the NADH₂-cytochrome-*c* reductase was normal (98). These nutritional studies were particularly important in light of the earlier work which specifically implicated vitamin E in these enzyme systems (99, 100).

The effect of the various lipids in the electron transport system on enzyme activity in different preparations of isolated enzyme systems has continued to yield conflicting reports. In most cases, the enzyme preparation was extracted with isooctane, presumably to remove non-polar lipid components from the preparation. Preparations treated in this manner have reduced cytochrome-*c* reductase activity. Some investigators (98, 101, 102, 103) have suggested that the inactivation was simply the result of the isooctane which remained in the preparation and that centrifugation or lyophilization to remove this excess solvent would reactivate the preparation. Other workers, however, have claimed further inactivation by centrifuging (104).

Yielding & Tomkins (105) have noted that progesterone inhibits the NADH₂-cytochrome-*c* reductase in rat skeletal muscle. This inhibition could be overcome by a variety of lipids, such as α -tocopherol, butyl stearate, menadione, or even butter.

Most investigators have continued to find the stimulating effect of tocopherol to be non-specific, since similar effects can be demonstrated by a number of compounds (101 to 107), although some claims have been advanced for specific reactivation by α -tocopherol (99, 100). Similar claims have also been made for specific reactivation by coenzyme Q, or its analogues, in the succinoxidase system (108, 109). Lipid factors, vitamin E or K₁, have enhancing effects on ubiquinone reactivation when inactivated by extraction with isooctane or acetone (108 to 110). However, cytochrome-*c* can replace coenzyme Q in the isooctane-extracted preparation, and it has been suggested that this may account for some of the discrepancies between laboratories. Napthaquinone inhibition of succinate oxidase activity can be reversed by ubiquinone although the latter does not function in a like manner with antimycin (111). Crane (112) has divided the ubiquinones and their analogues into four groups with respect to their ability to restore or to inhibit enzyme activity and the sensitivity of this activity to antimycin-A.

Since coenzyme Q, vitamin K, and α -tocopherol have similar structural features, methods employed for their quantitative determinations have, in the past, included all three compounds. Recent determinations, however, have emphasized the individualities of these compounds (113 to 117). All three compounds have been shown to be present in horse heart (114). In heart muscle mitochondrial preparations, ubiquinone is present in four to five times the concentration of the cytochromes (115). About 25 per cent of this ubiquinone is present in a bound form which can be extracted only after alkaline hydrolysis.

Green & Lester (79) outlined their reasons for assigning a coenzyme function to ubiquinone. There now appears to be agreement that antimycin-A inhibits ubiquinol oxidation (79, 80), but not ubiquinone reduction (118). The role of cytochrome-*c*₁ in ubiquinol oxidation is not clear (79, 119). The oxidation of coenzyme Q is said to be linked through cytochrome-*c*. However, Hatefi (119) has indicated that cytochrome-*c*₁ is not involved in the oxidation of coenzyme Q. He bases this opinion on the fact that when cytochrome-*c*₁ is added to a coenzyme Q oxidase system it does not stimulate oxidation.

When Redfearn & Pumphrey (80) investigated the kinetics of coenzyme Q oxidation and reduction, they showed that the rate of reduction was less than the rate of oxidation of either substrate, succinate or NADH, in heart muscle preparations. From these data, they have suggested that ubiquinone is not in the direct pathway of the respiratory chain but that it may function either as an interchain carrier or as an intermediate in oxidative phosphorylation. Wattenberg and co-workers (120, 121) have studied the effects of ubiquinone and menadione on tetrazolium reductase activity and concluded, on the basis of the ability of these compounds to enhance activity with different substrates, that they each act in specific dehydrogenase systems.

Chicks grown on a vitamin K-deficient diet were used in an investigation of the role of vitamin K in oxidative phosphorylation (122). To further inhibit vitamin-K activity, some of the animals were given sulfaquinoxaline, and the prothrombin time was evaluated to check the effect of this procedure. Even when, by this criterion, the vitamin-K activity was severely inhibited, the efficiency of oxidative phosphorylation was not affected with either glutamate or succinate employed as a substrate.

It has, however, been shown that treatment of bacterial extracts with ultraviolet light destroys a bound vitamin K-like compound (123). This photodestruction is accompanied by losses in both oxidation and phosphorylation, although the activity can be restored specifically by the addition of vitamin K₁ or closely related compounds (124). Vitamin K reactivated both oxidation and phosphorylation with a majority of the substrates tested. Both the rates of oxidation and phosphorylation almost tripled on addition of vitamin K, even when succinate was employed as a substrate. The authors (123), however, state that "with succinate as an electron donor, neither vitamin K₁ nor alpha tocopherol could reactivate," and they conclude that only NAD-linked pathways are reactivated. According to this account, succinate reactivation requires an unknown material present in the supernatant fluid.

The effects of dicumarol were also consistent with a role for vitamin K. At low concentrations, dicumarol was a competitive inhibitor of phosphorylation, and at high concentrations a non-competitive inhibition of oxidation was observed (124). With 2,4-dinitrophenol and thyroxine, there was a non-competitive inhibition of phosphorylation, but oxidation was not inhibited.

The enzyme system (125, 126) in rat liver homogenates, which catalyzes the reduction of neotetrazolium and employs succinate as a substrate, has been found to require a heat-stable cofactor. Of the materials tested, vitamin K₃ most resembles this cofactor.

Marki & Martius (127) have continued their studies on the role of vitamin K by isolating a vitamin-K reductase from beef liver. This FAD-containing enzyme is highly purified and appears to be homogeneous. It catalyzes the reduction of a whole series of quinones and can employ either NADH₂ or NADPH₂ as substrate. Wosilait (128) has purified a comparable enzyme from dog liver. In contrast to most of the similar enzymes, this enzyme is inhibited by dicumarol.

A number of schemes have been proposed for the mechanism by which vitamin K is activated in oxidation and phosphorylation (124, 129, 130). It has also been suggested that vitamin K may play a role in mediating hydrogen transfer from extra mitochondrial pyridine nucleotides to the mitochondrial chain (131).

Fatty acids themselves appear to affect oxidative phosphorylation. The ability of mitochrome (132) and a number of other preparations (133 to 137) to uncouple oxidative phosphorylation can probably be attributed to fatty acids.

It has been suggested (42) that the inactivation observed on isooctane extraction of enzyme preparations is attributable to the extraction of such un-

couplers. Electron transport in the extracted system is inhibited by the same factors that are involved in tightly coupled mitochondria, except that the means of transfer of the conserved energy may not be available in the enzyme preparation.

SUCCINIC DEHYDROGENASE

A number of colorimetric procedures for the assay of succinic dehydrogenase have been reported (138 to 141), and the nature of its flavin prosthetic group has been clarified (142). The flavin moiety is bound to the protein by covalent bonds and is liberated only after digestion by proteolytic enzymes. Even after purification, a peptide fragment is still attached to the liberated prosthetic group. This group appears to be a derivative of FAD, since it can be split at the pyrophosphate linkage to yield 5'-adenosine mononucleotide with a mononucleotide resembling, but not identical to, riboflavin-5'-phosphate. The results to date suggest that the isoalloxazine ring is present and connected to a ribityl chain but that the peptide chain may be directly connected to the flavin.

The soluble preparations (143, 144) of succinic dehydrogenase do not have properties of the endogenous form of the enzyme. The enzyme can, however, be reincorporated into heart muscle particles from which it has been removed. Once returned to the particle, it again has its original properties.

The substrate is oriented on the succinic dehydrogenase at cationic centers on the protein (145). The compound thus oriented is actually dehydrogenated by transelimination of hydrogen (146). This was demonstrated when fumaric and maleic acids were catalytically reduced with deuterium by using palladium, and the compounds thus obtained were then employed as a substrate for preparations containing succinic dehydrogenase. The fumarate resulting from the enzyme-catalyzed dehydrogenation of these substrates was then analyzed for its deuterium content, and results showed that the succinate was not dehydrogenated in a random manner but was clearly a transelimination.

Commoner & Hollocher (147, 148) have studied the free radicals produced in a succinoxidase preparation from heart muscle. Employing electron-spin resonance spectrometry, they showed that with succinate as a substrate the enzyme system produced organic free radicals which did not depend upon electron transport. By use of various inhibitors, it was determined that the radical was associated with succinic dehydrogenase. The data are compatible with the concept that the free radical is a form of the enzyme substrate complex.

PYRIDINE NUCLEOTIDES

One approach to an understanding of the role of pyridine nucleotides within the cell is to determine the amount and form in which they are present. The methods employed for the extraction and analytical determination of pyridine nucleotides were reviewed by Klingenberg & Bücher (149); the methods have been applied (150 to 161).

An important factor in these studies is the actual destruction of the nucleotides (158). Nicotinamide, which inhibits the destruction by NADase, has no effect on the state of oxidation or release of the pyridine nucleotides from mitochondria, and it can, therefore, be used to great advantage. Another important aspect is concerned with the manner in which the pyridine nucleotide is related to the integrity of the mitochondrial structure. The distribution of added coenzymes between the suspending fluid and the mitochondria is a function of the permeability of the mitochondria (159 to 161). A number of correlations have been made between the oxidation of reduced coenzymes and mitochondrial swelling. It appears that reduced coenzymes do not leave the mitochondria as readily as the oxidized compounds (163). Even in purified mitochondrial fragments, such as an NADH₂ oxidase, the pyridine nucleotide remains bound to the enzyme system. Added NADH₂ is not oxidized by exchanging with the enzyme-bound pyridine nucleotide (164).

The oxidation of NADH₂ by rat liver mitochondria can be stimulated four to six times by the addition of catalytic quantities of either acetoacetate or β -hydroxybutyrate. NADPH₂ oxidation is not stimulated unless some NAD is also present. Thus, it would appear that acetoacetate or β -hydroxybutyrate can function to transfer hydrogen from extramitochondrial pyridine nucleotide to intramitochondrial pyridine nucleotide by being alternately oxidized and reduced. Fresh tissue homogenates or fresh mitochondria, however, show essentially no β -hydroxybutyrate dehydrogenase activity (166) when assayed with NAD as an acceptor. Since swelling of the mitochondria causes a small increase in dehydrogenase activity with NAD as acceptor, it may be that β -hydroxybutyrate can function as indicated in preparations of modified mitochondria. Whether such a system functions *in vivo* is questionable.

Purvis (157) determined the amounts of pyridine nucleotides in mitochondria by fluorometric methods applied to acid and alkali extracts. Specific enzymes were used in order to make the analyses specific for the reduced and oxidized forms of the two pyridine nucleotides. It was found that after mitochondria was incubated with phosphate, ADP, or 2,4-dinitrophenol, the amount of NADP or NAD which reacted with alcohol dehydrogenase increased. This increase was not the result of oxidation of the reduced forms of the nucleotides. Since the author was convinced that his analytical procedures were reliable, he concluded that the extra NAD liberated during the incubation with phosphate, ADP, or dinitrophenol was caused by the release of NAD from some precursor form. Since the compounds which cause this liberation are connected with oxidative phosphorylation, it was postulated that an energy-rich intermediate of the pyridine nucleotide may be the precursor form.

Klingenberg & Slenczka (153) were unable to observe the "extra" NAD. However, the differences in observations may be attributable to the presence of an enzyme which catalyzes the liberation of NAD from the precursor form. Such an enzyme has been noted by Purvis (157) in commercial pre-

parations of alcohol dehydrogenase. In studies of the phosphorylation resulting from changes in the respiratory state of the mitochondrial pyridine nucleotides, a sudden increase in ATP formation was noted preceding the oxidation of pyridine nucleotide (167). The authors concluded that the reduced form of the pyridine nucleotide may be present in a high-energy intermediate form and that the sudden ATP increase is the result of the transfer from this intermediate form. The reduced pyridine nucleotide can then be oxidized.

At temperatures below 10° (168), reduced forms of the pyridine nucleotides of mitochondria slowly increase; NADH₂ accumulates more rapidly than NADPH₂ when these compounds are incubated with added substrate. It is only when the temperature is raised above 15° that oxidation of the coenzymes is demonstrated and that the rate of disappearance of NADPH₂ becomes greater than that of NADH₂. Succinate used as a substrate produced the highest amount of reduced coenzyme in mitochondrial suspensions (168, 169). Since succinate oxidation is not directly linked to pyridine nucleotides, these results suggest either that the nucleotides are reduced by systems with a higher potential or that energy consumed in chemical form can be utilized for reduction of the NAD. Dinitrophenol did, in fact, decrease the extent of the reduction of the coenzymes (168, 169) and, in aged mitochondria, succinate-linked pyridine nucleotide reduction was enhanced by the addition of ATP (77,338). These findings, which indicate that energy conserved at one portion of the electron-transport system can be used to drive reactions in another part of the system, are of considerable significance (77) and must be taken into consideration when evaluating the sites of energy conservation during the course of oxidative phosphorylation.

OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation is a complex process involving both the actual chemical mechanism by which energy is conserved in the electron transport chain and the mechanism by which this chemical energy is transferred to ADP to form ATP.

Many investigators approached the problems of oxidative phosphorylation through the mechanism involved in the transfer of the energy conserved in oxidation to ADP (170 to 177). Results of experiments employing inhibitors which affect flavin enzymes have led to the postulation that one of the energy-conserving steps in electron transport is at the flavin level and involves a flavin phosphate (174 to 177). The contraction and swelling of mitochondria have been studied as an index of the relationship of the respiratory and phosphorylative systems to structural integrity. Some reports have indicated that actual electron transport is required for swelling to take place (178, 179), whereas others have presented evidence that it is the state of the electron-transport components which is important in swelling (180, 181). Still a third group holds that phosphorylation, coupled to oxidation, is involved in the swelling phenomenon (182 to 184). It is probable that the most

important factor in controlling structural changes in mitochondria is the level of intramitochondrial ADP (185, 186, 187, 202).

The effect of the oxidative state of respiratory carriers on the ATP-ADP exchange has also been studied in an effort to determine whether the respiratory pigments are in the oxidized or reduced form at the time of chemical energy conservation (170, 188, 189).

The ultimate objective of all biochemical investigation is to take the data accumulated from isolated systems and apply them to the living cell. The opportunity to do this with precision is rare, but, with the technical advantages presented by their sensitive spectrophotometric techniques, Chance and co-workers (190 to 197; 199 to 201) have been able to accomplish this transition. Using a sensitive microspectrophotometer, it was possible to analyze components of the respiratory system and to determine their metabolic state in a single liver or kidney cell (190). It was even found possible to calculate the total concentration of cytochromes in the "Nebenkern" of the grasshopper spermatid and to show that the respiratory activity is associated with the mitochondrial bodies (191, 192). By fluorescence measurements, the pyridine nucleotides could also be localized in mitochondria (193, 204). Using the ascites tumor cell and the yeast cell, the efficiency *in vivo* of oxidative phosphorylation was compared accurately with the data obtained on an isolated system (194 to 196). In the intact cell, the phosphorylative efficiency is equal to the best values obtained with isolated mitochondria, and the sites (197) of phosphorylation, as determined by the so-called cross-over points, are the same as in the systems *in vitro*. The cross-over points themselves have been definitively established as existing between pyridine nucleotide and flavoprotein, cytochromes-*b* and -*c*₁, and cytochromes-*c* and -*a* (77). However, a cross-over point has been identified between flavoprotein and cytochrome-*b* in the rat liver mitochondria by employing choline as a substrate (203), and Ramirez (198) has accumulated data that an additional site may exist between cytochrome-*a*₃ and oxygen. This cross-over point is present only in the intact toad heart muscle and cannot be detected in isolated mitochondria (see also 339).

The phosphorylation associated with electron transport can be localized in certain portions of the respiratory chain by employing different substrates, electron acceptors other than oxygen, and various inhibitors. The phosphorylation which accompanies succinate oxidation has generally been held to result in a P/O ratio of 2. This has again been confirmed (205, 206). By employing cytochrome-*c* as the terminal electron acceptor, one phosphorylative step is localized between cytochrome-*c* and succinate (205), whereas the other exists between cytochrome-*c* and oxygen (207, 208). That this latter step is most labile has been suggested on the basis of assays of the phosphorylation resulting from ascorbate oxidation mediated by cytochrome-*c* (209 to 211). This does not appear to be a suitable assay of this phosphorylative step. A preparation which yields P/O ratios of close to 2 and 3 with succinate or β -hydroxybutyrate as a substrate, respectively, can give little or no phos-

phorylation with cytochrome-*c*-mediated ascorbate oxidation (207). Hence, if the cytochrome-*c* to oxygen span, common to all three substrates, is not producing a phosphorylation, the P/O ratio employing succinate or hydroxybutyrate should reflect the loss of this phosphorylation. It was concluded that extramitochondrial cytochrome-*c* can be oxidized by mitochondria without an accompanying phosphorylation, even though the phosphorylative mechanism is still intact. Complex anions and metal chelates (208) can mediate the mitochondrial oxidations of substrates which otherwise are not oxidized by these preparations. It has been suggested (208, 212) that these materials are not directly oxidized and reduced by these substrates, but that they may act by affecting structural features of mitochondria.

The control of respiration in the intact cell has been studied in various types of cells, with indications that this control is related to the level of ADP (200 to 202). The concentration of ADP is, in turn, a function of the concentration of the metabolites presented to the cell.

In the specific case of the Ehrlich ascites tumor cells, a number of studies employ improved procedures for the isolation of mitochondria (211, 213, 214, 215). Hawtrey & Silk (211) have reported that mitochondria isolated from such cells are deficient in NAD and indicated that this is attributable to NADase activity. Borst (213) suggests that the isolated mitochondria tend to lose cytochrome-*c*. Chance & Hess (216), on the basis of their spectrophotometric data, concluded that these cells have more than adequate amounts of the cytochrome chain, with very little, if any, of the cytochrome-*c* in cytoplasm (215).

Disrupted mitochondria and mitochondrial fragments have been made for the study of oxidative phosphorylation in order to eliminate some of the effects of the particulate membrane. Imamoto (209, 210) studied the effects of detergents on oxidative phosphorylation in rat liver mitochondria and found that cationic detergents inhibited oxidative phosphorylation to a lesser degree than non-ionic, anionic, and amphoteric detergents. Quantamin 24p, a cationic detergent, when used at low concentrations, greatly stimulated phosphorylation coupled with the oxidation of succinate in fresh mitochondria, but it accelerated the decrease in enzyme activity of aged mitochondria.

A number of investigations (187; 219 to 222) have implicated a soluble protein in oxidative phosphorylation. Linnane & Titchener (219) have indicated that a soluble protein plus magnesium can convert a non-phosphorylating electron-transport particle to a phosphorylating system. Lehninger & Gotterer (221) have isolated from mitochondria a soluble protein which carries the "contraction-promoting activity" and have named this the "c-fraction." Pinchot (222), working with his *Alcaligenes faecalis* system, which is a particulate NADH₂ oxidase system, a soluble heat-labile fraction, and polynucleotide, has shown that the system dissociates on incubation with NADH₂, but not with NAD. The soluble fraction, which is eluted by this procedure, can produce a net ATP synthesis when it is incubated with inor-

ganic phosphate and ADP. Thus, it would appear that in a number of systems, a soluble and presumably protein factor is involved in the coupling of the energy-conserving step of the oxidation system to the phosphorylation of ADP. This factor may itself be the element involved in the swelling phenomenon.

Chlorpromazine has been shown to inhibit electron transport between NADH_2 and cytochrome-*c* (223). This inhibition takes place only in phosphorylating systems and does not seem to affect the succinate cytochrome-*c* reductase system. It also appears to act competitively with cytochrome-*c* in the cytochrome-*c* oxidase step (224), and in both instances affects the coupled phosphorylation reactions. The effect of barbiturates on oxidative phosphorylation has also been investigated (225). The thiobarbiturates inhibit and, to a lesser extent, appear to uncouple oxidative phosphorylation, whereas the oxybarbiturates inhibit oxygen uptake. Aminoazobenzene derivatives (227) apparently inhibit respiration between NAD and cytochrome-*c*, probably more specifically at the flavoprotein-cytochrome-*c* reductase level. The effect of valinomycin is similar to that of dinitrophenol in uncoupling oxidative phosphorylation (228). Thomson & Sato (226) studied the effect of 13 inorganic phosphate compounds on oxidative phosphorylation, and only thiophosphate was found to be a true uncoupling agent.

The effect of thyroxine and closely related compounds (187, 217, 218, 229, 230, 231) has been studied on isolated particulate systems. Bronk (229), using an oxygen electrode in his studies of oxidative phosphorylation, was able to study the effect of different periods of incubation on mitochondrial and submitochondrial particles. There was an increased rate of both oxidation and phosphorylation with submitochondrial particles when succinate or NADH_2 were used at low concentrations of thyroxine. Similar results were obtained when mitochondria were treated with hypotonic phosphate buffer. At higher concentrations of thyroxine, oxidative phosphorylation was uncoupled.

Dallam & Reed (231) have also shown that thyroxine will result in increased oxidation of both NADH_2 and cytochrome-*c* by mitochondria, but they have attributed this to increased permeability to the substrates. Schole (217), on the other hand, studied the oxidation of cytochrome-*c* by a cytochrome-*c* oxidase preparation and noted that at pH 7.4 the rate of oxidation of the cytochrome is not increased by thyroxine, although it is accelerated at pH 8.5. This effect is significantly increased by preincubation of thyroxine with the cytochrome-*c* oxidase preparation.

It is not clear how these results correlate with the interesting findings of Dallam & Howard (339), which are as yet unconfirmed. These authors observed an enhanced phosphorylation with thyroxine over a very narrow concentration range. At these low concentrations of thyroxine, 10^{-5} M, there was no increase in the oxidation, but the thyroxine caused a tighter coupling of the phosphorylation. The results suggested that the increased phosphorylation took place in the span from cytochrome-*c* to oxygen, and the authors

indicated that not one but two phosphorylation sites may exist in this span (see also 198).

PEROXIDASE

Schultz & Rosenthal (232) have investigated the possibility that the porphyrin associated with preparations of myeloperoxidase may be the result of incomplete biosynthesis of the enzyme. They treated the enzyme preparation with ferrous sulfate to determine whether the iron would be incorporated into the porphyrin. On assay, it was found that, instead of increasing activity, it inactivated the enzyme, and further, that the inactivation depended on ferrous ion and oxygen. Sulfhydryl groups are not involved, since *p*-chloromercuribenzoate has no effect. This inactivation is relatively specific, since other metals and ferric ion do not inactivate the enzyme. The mechanism of the inactivation was not established, but, on the basis of spectrophotometric and other data, the authors suggest that the iron may be involved in an oxidative inactivation.

Mills (233) has presented evidence for an enzyme in erythrocytes which catalyzes the reduction of hydrogen peroxide to water and the oxidation of glutathione. The enzyme was termed a glutathione peroxidase, and its physiological role was pictured as protective in disposing of peroxide. Assays of rat liver, lung, heart, and kidney (234) have also shown these tissues to contain significant concentrations of the enzyme. The author has suggested that this enzyme may be coupled to oxidation of glucose-6-phosphate oxidation via the glutathione reductase system. Thus, glucose-6-phosphate would be oxidized to 6-phosphogluconate with reduction of NADP, which would in turn reduce oxidized glutathione. The reduced glutathione would react in the glutathione peroxidase system.

Most investigation centering on the enzyme tryptophan peroxidase or tryptophan pyrrolase is concerned with the fact that this mammalian enzyme appears to be induced. Tryptophan peroxidase activity in liver can be enhanced tenfold by injection of tryptophan into mammals. Nemeth (235) has followed the enzyme activity in fetal and postnatal stages up to adult in a number of animals. In all species studied, fetal liver contained very little of the enzyme, and the amount could not be increased by injection of tryptophan. The enzyme does increase rapidly from the low fetal level to the much higher adult level, but at differing times. Once the adult level of the enzyme is achieved, the injection of tryptophan produces a response.

Gordon and co-workers (236, 237) attempted to induce the production of the enzyme in a cell-free mammalian system, and they found an increase in preparations containing tryptophan-supplemented medium. The increased tryptophan peroxidase activity, however, was correlated with changes in the particulate and was not an actual increase of the enzyme. Fractionation of the cell particulate appeared to locate most of the enzyme in the microsomal fraction.

Greengard & Feigelson (238, 239), however, found that the enzyme was

confined to the soluble cell sap, whereas the microsomes contained an activator for enzyme activity. This activator had the characteristics of ferriprotohemin. These results suggested that the so-called induction of the enzyme is not the result of *de novo* synthesis of the protein but rather that the prosthetic group of the enzyme is released from the microsomes to the soluble phase of the cell. The importance of these findings is not limited to the fact that tryptophan peroxidase can no longer be considered an example of an inducible mammalian enzyme. Taken together with the findings of Rossi-Fanelli & Antonini (240), it may represent a more general phenomenon, that of translocation of heme prosthetic groups from one protein to another.

Hosoya (241 to 244) made an extensive study of turnip peroxidase, and, as in the case of numerous proteins, found at least three separable peroxidase fractions in crude extracts. The physicochemical properties of the three were very nearly the same except in electrophoretic mobility.

One of the turnip peroxidase fractions was crystallized, and its carbon monoxide, cyanide, azide, and fluoride complexes were spectroscopically similar to those of the enzyme obtained from horseradish. The molecular weight, determined on the basis of heme content or sedimentation and diffusion constants, was approximately 41,500.

All three enzyme fractions were compared kinetically to horseradish peroxidase; guaiacol and ascorbic acid were used as hydrogen donors. The rate constant determined for the turnip enzyme had the same order of magnitude as the horseradish enzyme when ascorbate was employed, but showed a thirtyfold difference for the crystalline protein when guaiacol was used as the substrate. The other two turnip enzyme fractions had lower constants than the horseradish enzyme.

By studying the effect of pH (244) on the kinetics of the reaction catalyzed by the turnip peroxidase, Hosoya (244) suggested that an α -amino group of the protein plays an essential role in the reaction of complex II with hydrogen donor molecules. This conclusion was strengthened in experiments in which the enzyme was treated with acetic anhydride, nitrous acid, and 2,4-dinitrobenzene sulfonic acid. The rate of reaction of complex II with the hydrogen donor after the enzyme had been treated was markedly decreased. From the results of treatment with the chemical reagents, the author was also able to conclude that imidazole and phenolic groups of the protein are not essential for enzyme activity.

Brill and co-workers (245) investigated the kinetics of the reaction between metmyoglobin and methyl hydrogen peroxide. They employed a rapid flow system with a sensitive magnetic susceptometer. Spectrophotometric data were obtained on the kinetics of the formation of ferrimyoglobin methyl peroxide compound under conditions of the magnetic experiments. A comparison of the magnetic and spectrophotometric measurements shows a difference in the kinetics of the reaction which increases at lower temperatures. The authors feel that the data are compatible with the production of a free radical. The value for the molar susceptibility of the compound which results

from the reaction between ferrimyoglobin and methyl hydrogen peroxide is that of a complex with two unpaired electrons and is in agreement with earlier values.

Yamazaki *et al.* (246, 247) have used paramagnetic resonance spectroscopy to study the mechanism of peroxidase activity. By use of hyperfine interaction, which gives rise to a fingerprint for free radicals, the authors identified the free radical under observation, as well as its concentration. By use of a flow technique, similar to the one employed in optical spectroscopy, they also identified the free radicals formed during the turnip peroxidase-catalyzed reaction. The free radical intermediates were derived from the substrates hydroquinone, ascorbic acid, and dihydroxy fumaric acid. In the steady state, the free radical depended upon concentration of the enzyme, substrate, and peroxide and was consistent with the mechanism shown in Figure 1.

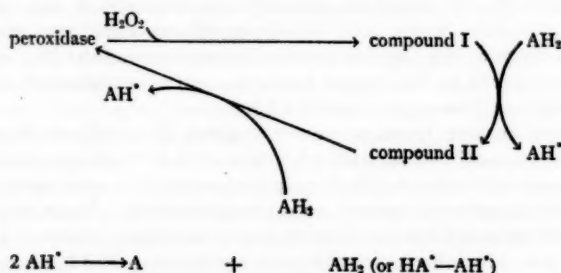


FIG. 1. Mechanism of peroxidase-catalyzed reaction.

In light of the evidence on the free radicals formed by the hydrogen donor in peroxidase reactions, it is not unexpected to find that phenolic compounds, such as thyroxine (248 to 250), phenolic estrogens (251), and quinones (252), can act as intermediates in peroxidase-catalyzed oxidations. As the mechanism in Figure 1 would indicate, when such compounds are employed as intermediates, they are themselves oxidized (251).

Peroxidases can exhibit oxidase activity in the presence of manganous ions (249, 251, 253, 254). Mudd & Burris (253, 254) found that cerous ions could also be employed. It would appear that peroxide generation is involved (249), since catalase will inhibit the activity of these systems.

CATALASE

The nature of the catalase molecule was studied by Samejima (255), who determined the diffusion and sedimentation constants of bovine liver catalase at different pH values and correlated these results with enzyme activity. The intact molecule at pH 7.0 has a molecular weight of 248,000. The molecule dissociates with increasing pH to an 85,000 molecular weight unit, and at pH

12 only this form is present. No catalase activity was present at pH 12, indicating that the low molecular weight unit was devoid of enzymatic properties. However, when the solution was brought back to neutrality, there was a partial restoration of enzyme activity. Sedimentation studies showed that two components were present, one corresponding to the intact molecule, and one which was considered a dimer of the smallest unit. These results suggest that the catalase molecule may be made up of three units, connected to form a rod-shaped molecule. It is not clear how these data relate to the fact that catalase is known to contain four heme groups per molecule, but a possible explanation is that calculations based on data obtained in strong urea solutions may be inaccurate.

3-Amino-1,2,4-triazole, which had previously been shown to depress dramatically the catalase activity of plants as well as that of liver and kidney in laboratory animals (256, 257), was found to act both as a reversible and an irreversible inhibitor of catalase *in vitro* (258). Margoliash & Novogrodsky (259) demonstrated that the irreversible inhibition reaction takes place only in the presence of H_2O_2 and results from a second-order reaction between aminotriazole, or a suitable analogue, and catalase- H_2O_2 complex I. This leads to the incorporation of one mole of C^{14} -labeled aminotriazole per mole of enzyme prosthetic group (258). The inhibitor apparently reacts with the protein moiety, since the heme group can be removed, leaving the inhibitor with the protein. The reaction of the inhibitor, however, does affect a portion of the protein in the vicinity of the heme group, since after reaction with triazole the hemoprotein will not react with cyanide. Kinetic data (260) have been interpreted as indicating that the reaction of one of the four hemes in the enzyme increased the reactivity of the others, probably representing the only form of heme-heme interaction thus far shown to occur in catalase. Hydrogen donors for catalase which compete with the inhibitor for complex I can prevent the irreversible inhibition reaction. This would explain how the normal occurrence of hydrogen donors in erythrocytes prevents the inhibition of erythrocyte catalase activity in animals treated with aminotriazole (261). This work clearly demonstrated the presence of ample hydrogen donors *in vivo* to react with available catalase- H_2O_2 complex I. It should be noted that none of the peroxidases (262, 263) investigated was inhibited irreversibly by aminotriazole, although it has been claimed that lactoperoxidase is reversibly inhibited by this compound (263).

Although it is frequently desirable to be able to assay catalase in the presence of peroxidase, or vice versa, it is often difficult to obtain accurate evaluation of the two enzymes. D-Cycloserine seems to offer some possibilities in this regard, since it markedly inhibits catalase at concentrations which will not inhibit horseradish peroxidase (264).

Higashi *et al.* (265) investigated highly purified erythrocyte catalase, employing both an enzymatic and an immunological assay. They prepared antisera to the catalase and applied the two assays to different preparations. The immunological assay gave higher results, which suggested that either more of

the apoenzyme than the heme enzyme was present or that some other inactive form of the enzyme was present in the tissue. This is reminiscent of the findings with tryptophan peroxidase (238, 239).

Catalase has been found to be distributed between particulate and extra-particulate, or cytoplasmic, fractions of the cell (266, 267). Injection of normal mouse tissue into normal mice caused a depression of catalase activity in both fractions of liver cells (267). Injection of Sarcoma 37 also depressed the total liver catalase, but it changed the distribution and more catalase was found in the particulate than in the cytoplasmic fraction. Adams (266) found that androgens will also bring about an intracellular redistribution of the catalase. De Duve and co-workers (268) have made a more detailed study of cell particulates and found that catalase is not associated with the mitochondria but with a particulate fraction which also contains amino acid oxidase and uricase activity. Thus, it would appear that catalase is located in a particulate with enzymes which produce peroxide, and its function within the cell seems to be clearly protective.

A number of investigators have indicated that there is a factor, present in both tumor and normal tissue extracts, which inhibits catalase *in vitro* (269 to 273). It would appear that these extracts simply contain substances which produce peroxide on auto-oxidation, thus leading to the formation of the inactive peroxide-catalase complex II (273).

The heterogeneity of the various dehydrogenases (279 to 291), such as glucose-6-phosphate dehydrogenase (279 to 281), lactic dehydrogenase (282 to 290), and malic dehydrogenase (291), has been found by many workers and has been interpreted in genetic and molecular terms.

Many of the apparently conflicting results which have been reported on mitochondrial enzymes can be attributed to mitochondrial structure (292). It now appears that flavins added to mitochondrial suspensions have no effect unless the mitochondria are damaged (292). The ability of the mitochondria to act on various substrates also depends upon the state of mitochondrial permeability (159, 160, 162, 165, 166, 293, 294). The enzyme activity depends upon the ability of the substrate to penetrate the mitochondria or the ability of the enzyme molecules to leak out of mitochondria (294). The various dehydrogenases are distributed differently within the mitochondria (295) and are, therefore, affected differently (294). It has been suggested that some of the apparently different NADH₂ dehydrogenases isolated from mitochondria may simply reflect the extent of mitochondrial fragmentation (296, 334, 335, 336).

GLUTAMIC DEHYDROGENASES

Glutamic dehydrogenase is a mitochondrial enzyme which appears to be under hormonal control (297). In concentrated solutions of the purified preparation, the enzyme polymerizes (298).

The enzyme does not appear to be highly specific, since it will oxidize other amino acids (304) and uses either NADP or NAD as an electron acceptor. Kubo *et al.* (298) have calculated that there are 15 binding sites on

TABLE II
RECENTLY DETERMINED STEREOSPECIFICITY OF PYRIDINE
NUCLEOTIDE-LINKED ENZYMES

Enzyme	Pyridine Nucleotide	Specificity	Reference
Glucose-6-phosphate dehydrogenase	NADP	β , II	274
6-Phosphogluconic dehydrogenase	NADP	β , II	274
Glutathione reductase	NADP, NAD	β , II	275
Glutamic dehydrogenase	NADP, NAD	β , II	276
Isocitric dehydrogenase	NADP	α , I	276
Xanthine oxidase	NAD	not stereo-specific	277
Mitochondrial NADH cytochrome- <i>c</i> reductase	NAD	β , II	277
Microsomal NADH cytochrome- <i>c</i> reductase	NAD	β , II	277
β -Hydroxysteroid dehydrogenase	NAD	β , II	278
3 α -Hydroxysteroid dehydrogenase	NAD	β , II	278
17 β -Hydroxysteroid dehydrogenase	NAD, NADP	β , II	278

this purified enzyme. Frieden (299) has shown that all of these binding sites are not the same and has designated one type as "active" and the other as "inactive." The active site is involved in the stoichiometry of the catalysis and can bind both NADH₂ and NADPH₂, whereas the inactive site is involved only in the dissociation of the enzyme molecule and does not bind NADPH₂.

Fisher (300) studied the kinetics of the formation of the enzyme-reduced coenzyme complex by spectrofluorometric measurements and correlated the kinetics of the complex formation with the kinetics of the over-all reaction. He established that the first step in the glutamic dehydrogenase reaction is the formation of the complex and that the increase in fluorescence is a direct measure of the complex. The addition of α -ketoglutarate causes a decrease in the fluorescence of the enzyme NADPH₂-glutamate complex. It would appear that α -ketoglutarate cannot combine with the enzyme itself but only with the enzyme-reduced coenzyme complex. A similar ternary complex (301) can be demonstrated with malic dehydrogenase, NADH₂, and D-malate.

Inagaki (302, 303) studied the denaturation of glutamic dehydrogenase by urea and bacterial proteinases. He found that NAD or glutamate protects the enzyme from these reagents, whereas NADH₂ accelerates denaturation. The same type of result was obtained for thermal denaturation, and, on this basis, the author concludes that the enzyme changes its stereochemical configuration on combination with substrate.

α -KETOGLUTARATE

Koike and co-workers (305) have purified the α -ketoglutarate dehydrogenation system from *Escherichia coli*. The system is a structural unit with a

molecular weight of 2 to 4 million and contains 11 moles of lipoic acid and 10 moles of FAD per mole of protein.

A fraction purified from an *E. coli* extract was found to contain NAD-linked dihydrolipoic dehydrogenase activity (306), whereas another fraction contained most of the enzyme activity resulting in oxidative decarboxylation with the formation of succinyl coenzyme A (307). The α -ketoglutarate dehydrogenase complex from hog hearts has now been separated into two fractions by both digestion with trypsin (308) and fractionation in the presence of 2 to 5 *M* urea (309). One of the isolated fractions contains the lipoyl dehydrogenase activity and is an FAD-containing flavoprotein, as is the enzyme purified from *E. coli*. Since the lipoyl dehydrogenase (310) does not contain any bound lipoic acid and is, nevertheless, inhibited by arsenite, a dithiol is implicated in the enzyme activity. Mechanisms for the reaction which involve free radicals have been presented (310, 311), but no signal could be detected in an electron paramagnetic resonance spectrum (311).

In contrast to these enzymes, Basu & Burma (312) have obtained from *Spinacia oleracea* dihydrolipoic dehydrogenase which is stereospecific and is active only with (-) dihydrolipoic acid. The sequence of electron transport in the complex is apparently: α -ketoglutarate \rightarrow bound lipoic acid \rightarrow FAD \rightarrow NAD (308).

XANTHINE OXIDASE

Most studies of the enzyme xanthine oxidase have attempted to clarify its mechanism of action. The source of some of the apparent discrepancies in these studies may be the presence of an inhibitor which is isolated along with the enzyme (313, 314).

The enzyme combines with one of the tautomeric forms of the purine substrate which does not necessarily have to be the major form present in solution (315, 316). The FAD prosthetic group of the enzyme is reduced on addition of the substrate according to first-order reaction kinetics (322).

Examination of xanthine oxidase frozen in liquid air by means of electron-spin resonance showed that free radicals are formed in the presence of substrate. The electron-spin resonance signal consisted of two peaks which could be obtained alone or in combination with each other. It thus appeared that there were two different sources of the free radical signal. One was presumed to be caused by an FAD-semiquinone free radical and the other by a reduced form of molybdenum (317).

Using chemiluminescent substances as indicators of free radical formation, it was shown that xanthine oxidase produced light in the presence of substrates and oxygen. The intensity of the light was directly related to the reaction velocity of the enzyme-catalyzed reaction. Similar observations were made with the free radicals initiated by sulfite oxidation, which results in oxygen consumption (318 to 320). The results indicate that the reduced enzyme is oxidized in more than one step with the production of free radical. It is suggested that these methods might form the basis for a general test for the presence of radicals.

Both the rate of reduction of the flavin and the rate of formation of uric acid in the presence of oxygen were studied in deuterium oxide solutions. A comparison of the rates of reduction of the flavin in water and deuterium oxide showed that this reaction was the same in both cases. However, the rate of formation of uric acid was much slower in the deuterium solution. It was concluded that this difference was attributable to a decrease in the rate constant for oxidation. Similar findings were also noted in experiments with free FAD and riboflavin-5'-phosphate reduced with dithionite (321, 322). The authors conclude that when hydrogens are replaced by deuterium atoms in the reduced flavin, the oxidation, which involves the removal of these two atoms, is affected, since the oxygen is bound to the reduced flavin across the reactive hydrogens prior to the oxidation step.

AMINO ACID OXIDASE

Meister *et al.* (323) reviewed some of the recent studies of the flavoprotein amino acid oxidases. L-Amino acid oxidases have been purified from the microsome fraction of chicken liver (324), from the venom of *Crotalus adamanteus* (325), and from cardin tuberculin (326). The venom preparation, which has a molecular weight of 133,000 and two moles of FAD per mole of enzyme, has been studied spectrophotometrically. During the course of these studies, it was noted that the introduction of small amounts of oxygen to the enzyme reduced by dithionite or substrate caused a spectral shift with maxima at 375, 385, and 475 m μ (327). Introducing additional oxygen resulted in a spectrum characteristic of the usual oxidized flavoprotein. The spectral shift was not associated with peroxide, ammonia, amino acids, or α -keto acids. The authors interpreted these findings as evidence of an intermediate complex between reduced flavoprotein and oxygen, and suggested that the reoxidation of flavoprotein by oxygen is at least a two-step reaction. Kubo *et al.* (328), working with D-amino acid oxidase, also noted an intermediary form of the FAD-containing enzyme but with an absorption maximum at 565 m μ .

Crystalline preparations of D-amino acid oxidase have been described (329, 330), and the nature of the binding sites of the apoenzyme for the FAD prosthetic group has been investigated (331, 332). Two binding sites were distinguished, one of which appears to be sulfhydryl groups of the apoprotein which combines with the AMP moiety of FAD. The other site concerns the binding point of the riboflavin-5'-phosphate portion of the FAD. A kinetic analysis of the interaction of the apoenzyme with various compounds suggests that these two binding sites interact.

A study (333) of the competitive inhibition of D-amino acid oxidase by benzoate has suggested that this inhibitor can react with the enzyme in two different ways. Benzoate can form an enzyme inhibitor complex directly, or it can react with an enzyme substrate complex to yield an enzyme-inhibitor complex.

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CHEMISTRY OF THE CARBOHYDRATES¹

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Since the previous review (1) dealt mainly with di-, oligo-, and polysaccharides, this review emphasises the monosaccharides. The extent of the carbohydrate field may be assessed from the number and variety of review articles which have appeared in the past year. The marked recent expansion of interest in amino sugars has resulted in numerous publications, and several aspects of the field have been reviewed, including the organic chemistry of amino sugars (2, 3), heparin (4), and chitin (5). Amino sugars are components of many bacterial polysaccharides (6). Various aspects of polysaccharide chemistry have been reviewed, including structure (7) and structural methods (8), immunological specificity (9), polysaccharides of microorganisms (6), alginates (10), hemicelluloses (11), starch (12) and its molecular properties (13), alkaline decomposition of polysaccharides (14), cellulose (15), dextran (16), and polysaccharides of Gram-negative bacteria (17). A variety of other carbohydrate topics have been surveyed: photosynthetic intermediates (18), catalytic reduction (19), stereochemistry (20), D-glucose (21), browning (Maillard) reactions (22), cyclitols (23), ion-exchange chromatography of sugar derivatives (24), β -glucuronidase (25), glycosyl thiocyanates (26), physicochemical properties of carbohydrates (27), carbonates (28), biosynthesis of aromatic compounds from glucose (29), and nonulosaminic acids (30).

PHYSICAL METHODS

More extensive use is now being made of various physical methods in carbohydrate chemistry, but their impact has hardly yet been felt.

Vapour-phase chromatography.—Vapour-phase chromatography of carbohydrates has been limited by a lack of suitably volatile derivatives. Recent results indicate a potentially wide application of the method. Trimethylsilyl (trisyl) ethers of sugars are sufficiently volatile (31), and, moreover, the parent carbohydrates are recoverable simply by boiling the trisyl ethers with water. Trisyl ethers of various sugars and uronic acids have been prepared (32, 33, 34). Certain *O*-isopropylidene derivatives of sugars may be separated, such as a mixture of 6-deoxy and 5-deoxy derivatives of 1,2-*O*-isopropylidene-D-glucose (35). Mixtures of various methylated sugars may be separated either as their methyl glycosides or as the acetylated itols (36, 37).

Optical rotation.—Hudson's isorotation rules must be modified for sugar derivatives that contain highly polarisable aglycones, for example, halogen atoms (38). A new, empirical, general correlation of molecular rotation and absolute configuration of sugar derivatives has been enunciated (39); it is of

¹ The survey of the literature pertaining to this review was concluded in September, 1960.

wider application than previous rules. Yamana (40) has also examined in detail the molecular rotation of glycosides as a function of structure. Rotatory dispersion studies have permitted an interpretation of the behaviour of solutions of methyl cellulose (41).

Infrared spectroscopy.—This method permits a differentiation between γ - and δ -lactones of aldonic acids (42) but not the identification of 1,3-dioxolan rings in cyclic acetals of carbohydrates (43). Infrared and Raman spectra of 1,3-dioxolan and certain derivatives (44) and of a variety of steroid glucosiduronates have been examined in detail (45). On the basis of certain absorptions exhibited by glycosides of known conformation, the conformations, hitherto uncertain, of other glycosides have been allocated (46). Infrared spectroscopy has been used to deduce that the aldehyde groups in periodate-oxidised cellulose are variously modified but mainly by conversion to hemiacetal groups (47). Parker (48) has described a valuable method for determination of the infrared spectra of sugars in aqueous solution and so measured the mutarotation constants (48). The infrared spectra of a series of polysaccharides elaborated by strains of *Cryptococcus neoformans* have been examined in detail (49). Glycopyranosides, in which the hydrogen at the glycosidic centre is replaced by deuterium, are valuable compounds for the interpretation of the infrared spectra of non-deuterated compounds (50).

Mass spectrometry.—Using the electron impact method, sugars have been shown, mass spectrometrically, to undergo fission predominantly at the glycosidic centre (51). The bond dissociation energy of β -glycosidic links is greater than that of the α -anomers. Methylated laminarin gave a series of fragments arising from disruption of individual sugar units and from step-wise fragmentation of the polysaccharide (52).

2-Oxo-L-"gulonic" acid has been shown, by polarography, to exist in the cyclic hemiacetal form (53).

OXIDATION OF CARBOHYDRATES

Although much is known about periodate oxidation (1), interesting new facts are continually being brought to light. Sodium metaperiodate is soluble in *N,N*-dimethylformamide, but glycol cleavage does not proceed unless water is added, and the percentage of water added controls the reaction rate (54). Thus, the use of aqueous *N,N*-dimethylformamide solvent mixtures may retard fast periodate oxidations to a speed suitable for kinetic studies.

Whereas L-threitan (1,4-anhydro-L-threitol) reacts with periodate and lead tetraacetate, 1,6-anhydro- β -D-glucofuranose and 1,6-anhydro- β -D-galactofuranose, which contain an apparently similar structural unit, are resistant (55). The view that this resistance is due to the molecular rigidity of the bicyclic systems preventing the formation of intermediate cyclic complexes is supported by work on the camphan-2,3-diols (56). The 2-*endo*-3-*exo*-, and 2-*exo*-3-*endo*-diols are markedly resistant toward periodate, lead tetraacetate, and phenyl iodosoacetate at room temperature but react slowly at elevated temperatures (50°–80°). The projected, or dihedral, angle be-

tween the two hydroxyl groups in the camphane diols is 120° . Examples in the steroid field, important for mechanistic studies, have been recorded by Angyal & Young (57), who observe that the formation of an intermediate cyclic complex is apparently mandatory for periodate oxidations but not for glycol cleavage by lead tetraacetate and phenyl iodosoacetate. Thus, the latter reagents, but not periodate, will attack *trans*-decalin-9,10-diol; it is difficult to imagine the formation of an intermediate cyclic complex in this case. Periodate attacks $\alpha\beta$ -diols by an electrophilic mechanism, whereas a nucleophilic mechanism is involved with $\alpha\beta$ -diketones (58).

The different steric requirements of periodate and lead tetraacetate in carbohydrate glycol cleavage reactions is illustrated with sucrose (59). With a limited amount of oxidant, the glucopyranose moiety is preferentially attacked by periodate, whereas the fructofuranose moiety is selectively cleaved by lead tetraacetate. The periodate reactions are as would be expected if the formation of a cyclic periodate complex were involved, since the projected angles in the glucose diol units are 60° or less and, hence, more amenable to complex formation than the fructose $C_3:C_4$ -diol unit where the projected angle is near 120° . This difference in steric requirements is seen with simpler compounds; thus, cyclopentan-*trans*-1,2-diol reacts much more rapidly with lead tetraacetate than does cyclohexan-*trans*-1,2-diol. One of the two aldehyde groups formed on oxidation of nucleosides with periodate may be selectively reduced by sodium borohydride in acid media (60).

Oxidations of carbohydrates by lead tetraacetate (61) and periodate (62) have been reviewed.

Periodate oxidation of acyclic sugar derivatives in unbuffered solutions may be complicated by the formation of cyclic intermediates. At pH 1 or 7.5, 3-*O*-methyl-D-glucitol (63) reacted with periodate to yield methoxymalondialdehyde, which underwent further oxidation. However, at pH 3.5, oxidation was incomplete, and it was suggested that 50 per cent of the 3-*O*-methyl-D-glucitol was selectively cleaved (64) at C_5-C_6 to yield an aldehyde sugar, which converted to a pyranose derivative and then was further oxidised to yield 4-*O*-formyl-2-*O*-methyl-L-threose (Figure 1). The stability of the formate ester prevented further oxidation. Related results were encountered in the periodate oxidation of 2-acetamido-2-deoxy-3-*O*-methyl-D-glucitol (65). D-Mannose phenylhydrazone and D-xylose 2,4-dinitrophenylhydrazone react in the acyclic form with periodate (66).

"Over-oxidation" with periodate involves hydroxylation of malondialdehyde or its derivatives with subsequent further glycol cleavage reactions. A mechanism for the hydroxylation has been postulated (67). The presence of an active methylene group is not the sole molecular feature that controls hydroxylation; rather, the presence is required, on 1,3-carbon atoms, of hydroxyl groups, one of which must be provided by an enolisation process. This explains why ethyl acetoacetate, cyanoacetic acid and diethyl malonate are not oxidised by periodate (68). The hydroxylation of malondialdehyde may involve the formation of a cyclic complex between periodate and the

hydrated enol form. Marder & Schuerch (69) find that malondialdehyde consumes 4 moles of periodic acid yielding 2 moles formic acid and 1 mole of carbon dioxide. These results are significantly different from those recorded by Heubner *et al.* (68). Paralleling the above pattern, 2-deoxy-D-"glucose" was found to consume 6.7 moles of periodic acid liberating 4 moles of formic acid and 0.82 moles of carbon dioxide. Different results have been obtained for the oxidation of 2-deoxy-D-"glucose" and 2-deoxy-D-"galactose" with sodium metaperiodate (70). Oxidation of the hydroxy malondialdehyde to mesomaldialdehyde may proceed by way of a five-membered cyclic complex

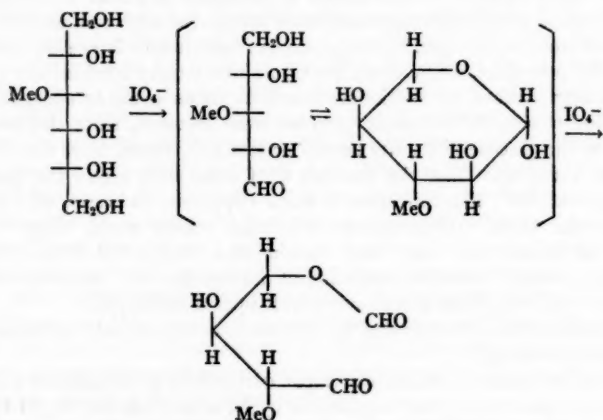


FIG. 1. Oxidation of 3-O-methyl-D-glucitol with periodate at pH 3.5.

(67) between periodate and the enediol form of the dialdehyde. A different mechanism, involving addition of periodate to the double bond in the enediol moiety, may explain a similar reaction stage in the oxidation of reductinic acid (71).

It is abundantly clear that periodate oxidation is not a simple, unequivocal analytical method. The subtleties in the variation of reaction patterns attendant on change of pH and oxidant concentration create pitfalls but also permit the acquisition of valuable data.

A satisfactory general method for the oxidation of secondary hydroxyl functions to ketones in cyclic derivatives of carbohydrates would be of considerable value but still remains to be found. Oxidation methods, which work well in the alicyclic field, are singularly unsuccessful with carbohydrates. The oxidation of secondary hydroxyl functions in acyclic carbohydrates, or in acyclic side chains of cyclic carbohydrates, is easily accomplished. For example, methyl 2,3-O-isopropylidene-L-rhamnofuranoside is converted to the 5-oxo derivative by pyridine-chromium trioxide (72), and

3,4-*O*-isopropylidene-1,5-di-*O*-trityl-D-arabinitol yields the 2-oxo compound with chromium trioxide in acetic acid (73). Oxidation of 1-deoxy-1-*S*-ethyl-D-arabinitol with *Acetobacter suboxydans* affords 5-deoxy-5-*S*-ethyl-D-threopentulose in which the keto function is not involved in hemiacetal formation (74). Acyclic carbohydrates with free keto functions may, of course, be obtained by the action of diazomethane on acylated aldonyl chlorides; a recent example is 1,3,4,5-tetra-*O*-benzyl-D-erythropentulose (75). Marei & Raphael (76) have described an interesting indirect synthesis of an oxo derivative of a substituted glycerol.

Reaction of methyl β -D-glucopyranoside with a dichromate-oxalic acid mixture (77) gave, *inter alia*, a small yield (ca. 0.5 per cent) of the methyl 3-oxo- β -D-"gluco"-pyranoside (III; see Figure 5, page 54).² In general, reduction of the oxo compound and identification of the resultant glycosides serve to locate the keto function. Thus, the 3-oxo compound (III; see Figure 5) yields methyl β -D-allopyranoside in addition to methyl β -D-glucopyranoside. Improved, though still low (4 per cent), yields of the 3-oxo compound result from the action of chromium trioxide in acetone on methyl 6-*O*-trityl- β -D-glucopyranoside (78) and methyl 4,6-*O*-ethylidene- β -D-glucopyranoside (79). Small amounts of the 2-oxo and 4-oxo compounds are also obtained in the former reaction. The oxo-compounds may be isolated by way of their bisulphite complexes and charcoal-Celite chromatography. The more ready complexing of the 2-oxo than of the 3-oxo compound with bisulphite has been explained on conformational grounds (80). Oxo compounds are also formed (81) in the oxidation of methyl β -D-glucopyranoside with chlorine or hypochlorite in the pH range 3 to 10. Chromate-oxidised cellulose contains 3-oxo-D-"glucose" units, since D-allose was obtained after reduction and hydrolysis; 2-oxo-D-"glucose" units are formed to a lesser extent (82). The presence of 2-oxo-D-"glucose" units has been demonstrated by other means (83), as have 2,3-dioxo units (84).

β -Glucosidase does not attack the oxo-"glucosides" (85). They are also sensitive to acids, yielding, *inter alia*, reductic acid and furfural. Reduction of methyl 2-oxo- or 3-oxo- β -D-"gluco"-pyranoside yields methyl β -D-glucopyranoside (equatorial epimer) or glucopyranosides in which the hydroxyl group derived from the keto function is axial (axial epimers). Steric hindrance in the reduction process leads to the formation of axial epimers. Reduction of the 2-oxo and 3-oxo compounds with hydrogen/platinum, hydrogen/nickel, sodium amalgam, and sodium borohydride gave, in each case, a mixture in which the axial epimers predominated to a greater or lesser extent. Methyl 3-oxo- α -D-"gluco"-pyranoside, in which the keto function is hindered by the axial glycosidic methoxyl group, gave a much higher per-

² Strict carbohydrate nomenclature would designate the 2-oxo derivative as methyl 2-oxo- β -D-arabinohexopyranoside, the 3-oxo derivative as methyl 3-oxo- β -D-ribohexopyranoside and the 4-oxo derivative as methyl 4-oxo- β -D-xylohexopyranoside. Generally in this review the name of a sugar within quotation marks implies chemical derivation from that sugar with loss of an asymmetric carbon.

centage (ca. 90 per cent) of methyl α -D-allopyranoside (axial 3-epimer) than did the β -anomer on reduction with sodium borohydride and hydrogen/platinum (86). Under similar conditions, *epi*-inosose-2, in which the keto function is hindered by an axial β -hydroxyl group, is reduced to yield the axial epimer only (87).

Methyl 3-oxo- β -D-"gluco"-pyranoside affords an oxime which on reduction with hydrogen/platinum gives an 85 per cent yield of methyl 3-amino-3-deoxy- β -D-allopyranoside (axial 3-epimer), whereas with sodium amalgam an almost equimolar mixture of 3-epimers results (88). There is, thus, a parallel with the reactions of inosose derivatives (89). The conversion of oxo derivatives into amino sugars constitutes a valuable additional method of amino sugar synthesis (2, 3).

The hydroxymethyl groups in glycopyranosides may be selectively oxidised to carboxyl functions by various methods (90), including catalytic oxidation by which D-glucosaminuronic and D-galactosaminuronic acids have been synthesised (91). A novel application of selective oxidation in polysaccharide structural determination has been described by Aspinall *et al.* (92). Graded acidic hydrolysis of an arabinoxylan from rye flour selectively cleaves the arabinofuranose side chains from the xylan. The arabinofuranose-xylose linkage may be stabilised by selective oxidation of the hydroxymethyl groups in the arabinofuranose moieties. On acidic hydrolysis of the oxidised polysaccharide, the anticipated acid-stable aldobiouronic acid was obtained and shown to be 3-(arabinofururonosyl)xylose. The method has also been applied to larch ϵ -galactan (93). The reverse procedure has been described by Smith & Stephen (94). Carboxyl groups are reduced by diborane in preference to ester groups, and acetylated pectic acid was thus converted to a galactan. Mesquitic acid acetate and alginic acid propionate were reduced likewise. Both methods will no doubt find extensive application in the carbohydrate field. A pseudoaldobiouronic acid, 2-(α -D-glucopyranosyl)-D-glucuronic acid, has been synthesised enzymically and found to be readily hydrolysed with acid (95).

The sugar moiety hydroxymethyl group in ribo- and deoxyribonucleosides is converted to a carboxylic acid function by platinum-catalysed oxidation (96). This reaction is a key step in a projected stepwise degradation of polynucleotides.

D-Glucose may be degraded to D-arabinose in 35 per cent yield by hypochlorite; at pH 11 D-glucose is converted to D-gluconic acid, and at pH 4.5 to 5.0 the acid is degraded to D-arabinose (97). β -D-Glucose is rapidly oxidised by bromine at pH 5 to D-glucono- δ -lactone (98), whereas, contrary to previous belief (99), the α -anomer is effectively inert and anomerization must precede oxidation. The reaction of α -D-glucopyranose would involve the formation of an axial hypobromite group, the formation of which presumably is sterically hindered. Previously reported oxidation rates for α -D-hexoses are probably their rates of anomerization (100).

A wide variety of carbohydrates is attacked by manganese dioxide (101);

for example, hexoses are degraded to pentoses and glyconic acids; 1→3, 1→4, and 1→6 linked D-glucose disaccharides are degraded to the corresponding D-glucosyl-D-arabinoses. The oxidation of D-glucose by cupric salts has been examined kinetically (102).

DEOXY SUGARS

A variety of deoxy sugars are now known to occur naturally, and the interest in this group of compounds is reflected by the search for new methods of synthesis. Reduction of methyl 3-oxo- β -D-"gluco"-pyranoside with platinum-hydrogen in acid media (103) yields methyl 3-deoxy- β -D-"gluco"-pyranoside in addition to methyl β -D-glucoside and allopentose (88). Treatment of 1,2-O-isopropylidene-5,6-di-O-tosyl- α -D-glucose with sodium iodide in acetone gave 1,2-O-isopropylidene-4-vinyl- α -D-xylotetrofuranose (104). Hydroboration of the vinyl compound with sodium borohydride and aluminium chloride, decomposition of the borane derivative with alkaline hydrogen peroxide, and acidic hydrolysis gave 5-deoxy-D-"glucose." Reduction of 5,6-anhydro-1,2-O-isopropylidene- α -D-glucosylfuranose with hydrogen and exhaustively deionized Raney nickel followed by acidic hydrolysis of the product gave 5-deoxy-D-"glucose" in addition to the normal reaction product, 6-deoxy-D-glucose (35). All the monodeoxy derivatives of D-glucose are now known to be crystalline. The β -pyranose and α -furanose forms of 3-deoxy-D-"glucose" have been prepared (105). 1,2:5,6-Di-O-isopropylidene-D-glucosylfuranose is converted to a 3-deoxy-3-iodo derivative by triphenylphosphite methiodide and thence to a 3-deoxy derivative by hydrogenation (106). Derivatives of 2-deoxy- β -D-"ribo"-furanose have been obtained by treatment of methyl 2,3-anhydro- β -D-ribofuranoside with sodium ethyl sulphide (107). Episulphide intermediates were postulated in certain of these reactions. Carbohydrate episulphides have now been prepared and their properties examined (108).

A variety of deoxy sugars has been prepared by conventional methods, viz., 2-deoxytetroses (109), 2-deoxy-D-"ribose"-1-¹⁴C (110) and, certain derivatives (111), 5-deoxy-D-arabinose (112), 3-deoxy-D-"mannose" (113), 2,5-dideoxy-D-"ribose" (114), and 5,6-dideoxy-L-arabinohexose (115). 3,6-Dideoxy sugars have already been reviewed (1). Chemical (116, 117, 118) and enzymic syntheses (119) of the biochemically important 2-deoxy-D-"ribose" 5-phosphate and 2-deoxy-D-"ribo"-furanose 1-phosphate (120) have been described. Several acylated derivatives of 2-deoxy-D-"ribose" have been described (121). The glycosyl chloride (122) obtained from the anomeric 1,3,5-tri-O-p-nitrobenzoates has been used in a synthesis of 2'-deoxyadenosine (123). Thus, the last outstanding step in the direct chemical synthesis of D-ribose and 2-deoxy-D-"ribose" nucleosides and nucleotides is completed. 6-Deoxy-D-glucose is a component (124) of holothurin A, the toxic principle of *Actinopyga agassizi* (sea cucumber).

Bines & Whelan (125) have succeeded in converting most of the D-glucose units in potato amylose into 6-deoxy derivatives, and they find that the

modified polysaccharide is still degraded by salivary α -amylase. Similar results were obtained when a proportion of the amylose D-glucose units were converted to 3,6-anhydro derivatives.

REACTIONS OF CARBOHYDRATES IN ALKALINE MEDIA

The alkaline degradation of reducing carbohydrates to yield meta- and isosaccharinic acids is considered (126) to proceed by β -alkoxy eliminations to yield α -dicarbonyl intermediates which undergo benzilic acid-type rearrangements (Figures 2 and 4). These reactions are being increasingly applied in the linkage analysis of saccharides, since the nature of the degradation products is indicative (127) of the point of substitution of a reducing sugar. Clearly, a full understanding of the reaction mechanisms is desirable if

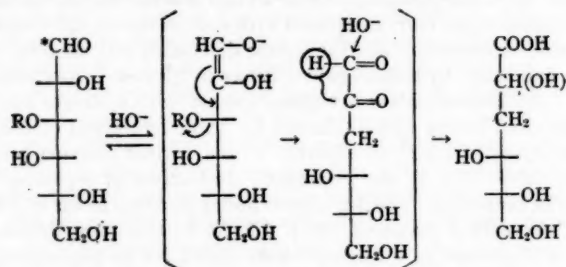


FIG. 2. Reaction of 3-substituted derivatives of D-galactose with alkali.

maximum information on structure is to be gained from a particular alkaline degradation; recent work has been of value in this respect. The occurrence of intramolecular rearrangements and fragmentation-recombination reactions may be deduced by using C^{14} -labelled compounds. D-Galactose-1- C^{14} was converted (128) by lime water to α -D-galactometasaccharinic acid in which C_1 contained 95 per cent of the original radioactivity, as would be expected for the reaction sequence shown in Figure 2, which involves an intermediate H migration. On the other hand, the behaviour of D-glycerose-3- C^{14} in alkali depends on the cations present (129). Thus, as shown in Figure 3, sodium hydroxide promotes H migration in the benzilic acid rearrangement of the α -dicarbonyl intermediate, whereas lime water causes both H and CH_2 migration.

Treatment of lactose-1- C^{14} with lime water gave α -D-glucosaccharinic acid in which 94 per cent of the original radioactivity was located in the C_2 -hydroxymethyl carbon atom, again indicative of an intramolecular rearrangement and the reaction sequence in Figure 4. However, the α -D-glucosaccharinic acid (II) obtained by the action of alkali on mannose-1- C^{14} (I) contained 39 per cent of the original radioactivity in the C_2 -methyl carbon atom and 57 per cent in C_2 itself. Clearly, a fragmentation-recombination

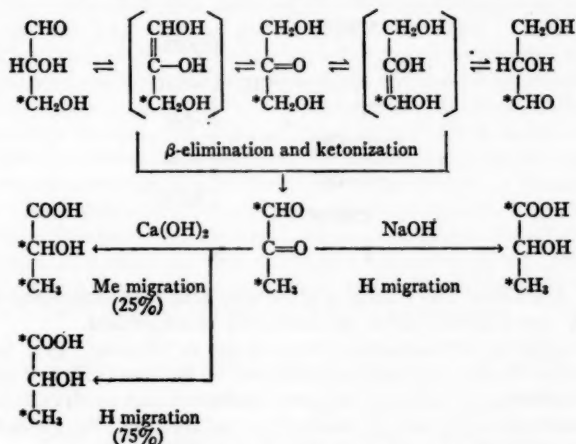


FIG. 3. Reaction of glyceraldehyde with alkali.

reaction has occurred in this reaction, but the precise mechanism is not apparent. α -D-Glucosaccharinic acid has been shown to be 2-C-methyl-D-ribonic acid (130).

Certain α -dicarbonyl intermediates have now been isolated. The first of these (131) was from methyl 3-oxo- β -D-"gluco"-pyranoside (III; see Figure 5) which is very sensitive to alkali, and its rapid rate of decomposition (Figure 5) resulted in a build-up of the α -diketo intermediate (IV), which was

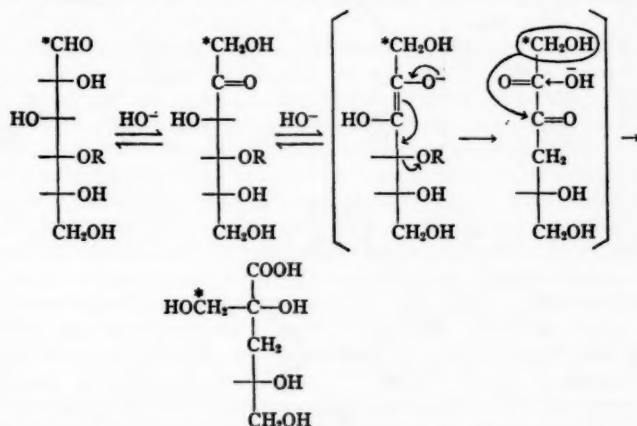
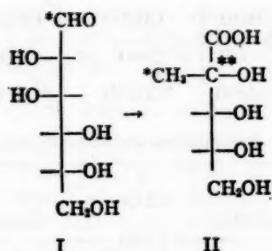


FIG. 4. Reaction of 4-substituted derivatives of D-glucose with alkali.



isolated crystalline. The products of benilic acid rearrangements of Compound IV (see Figure 5) have not been fully characterized.

The α -diketo intermediate, 4-deoxy-2-oxo-D-"fructose" (V), has been isolated after the action of sodium hydroxide on maltose (132) and cellobiose (133); its structure is indicated by peroxide degradation to glycollic acid and D- β - γ -dihydroxybutyric acid. 4-Deoxy-2-oxo-D-"fructose" (V) yielded 90 per cent of isosaccharinic acids with lime water (Figure 4), whereas sodium hydroxide attacked the α -diketo compound more slowly and yielded, *inter alia*, glycollic acid and D- β - γ -dihydroxybutyric acid in addition to isosaccharinic acids. The benilic acid rearrangement of 4-deoxy-3-oxo-D-"fructose" was considered to be catalysed by $(\text{CaOH})^+$.

The α -dicarbonyl intermediate, 3-deoxy-D-"glucosone" (VI) has been obtained after treatment of 3-O-benzyl-D-glucose with sodium hydroxide

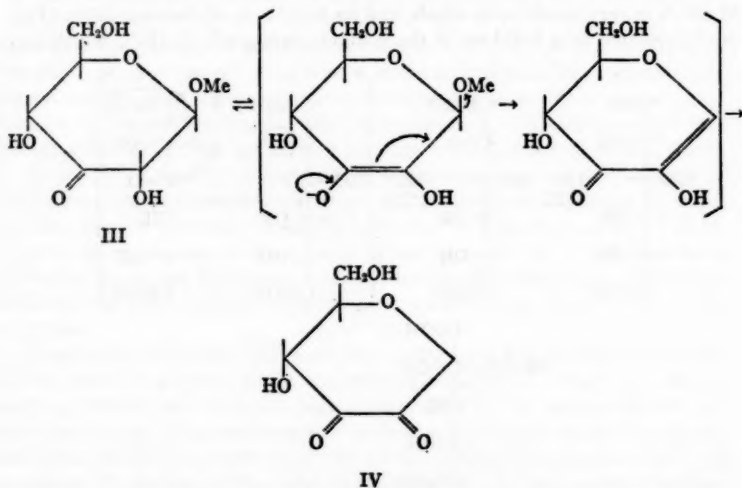
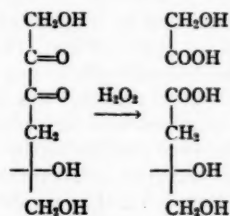
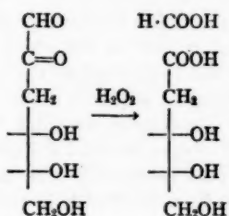


FIG. 5. Behaviour of methyl 3-oxo- β -D-"gluco"-pyranoside with alkali.

(134). The *erythro* and *threo* forms of the dicarbonyl compound have also been prepared from diketose glycine derivatives (135). The 3-deoxy-D-"glucosone" yielded (134) formic acid and 2-deoxy-D-"ribonic" acid with hydrogen peroxide, and with both lime water and sodium hydroxide it was converted almost quantitatively into metasaccharinic acid (cf. Figure 2). The reaction was more rapid with the former reagent, which also gave a higher proportion of α -metasaccharinic acid. The β -alkoxy elimination apparently is the rate-determining step in the alkaline degradation in Figure 2. The benzilic acid rearrangement of glyoxal to glyoxylic acid in 0.005 *N*-sodium hydroxide at 0° is catalysed (136) by cations in the sequence $\text{Ca} > \text{Tl} > \text{Ba} > \text{Li}$. The benzilic acid rearrangement of diacetyl has also been examined (137). Thus, the long-known effect of calcium in the alkaline degradation of carbohydrates is due to catalysis of the benzilic acid rearrangement.



(V)



(VI)

Alginic acid, which contains D-mannuronic and L-glucuronic acid units, is degraded by sodium hydroxide (138) to yield a mixture of 3-deoxy-2-C-hydroxymethylpentaric acids, thus confirming that the polysaccharide is predominantly 1 \rightarrow 4 linked.

Under appropriate conditions, D-glucose and D-fructose are converted to a mixture of DL- and D-sorbose by potassium hydroxide or by a strongly basic anion exchanger. The distribution of radioactivity in the products obtained by isomerizing D-glucose-1-C¹⁴ indicates (139) that a 3,4-enediol is an intermediate and that a fragmentation-recombination reaction does not occur.

Whilst alkyl glycosides are usually considered to be alkali-stable, the presence of certain structural features may cause certain arylglycosides to be alkali-labile (140). Thus, phenyl- β -D-glucopyranoside is converted to 1,6-anhydro- β -D-glucose by way of an intermediate 1,2-epoxide. The intermediate can form only if the C₂-hydroxyl group and glycosidic phenoxy group are *trans*-disposed; hence, phenyl α -D-glucopyranoside is alkali-stable. If a 1,6-anhydro ring cannot form, then transglycosylation may occur (141). Phenyl β -D-xylopyranoside affords methyl β -D-xylopyranoside with sodium methoxide in methanol, and β -D-xylopyranosyl, 1'- and 2'-glycerol with sodium and glycerol. Treatment of cellulose or amylose with alkaline aqueous phenyl β -D-xylopyranoside effected the addition of xylose units to the poly-

saccharide. It is surprising that this type of reaction has not been exploited further. Under the alkaline conditions used in cellulose purification, alkyl glycosides partially decompose (142). Thus, with 10 per cent sodium hydroxide at 170°, cellobiitol and lactitol (143) afford 1,6-anhydro- β -D-glucose and 1,6-anhydro- β -D-galactose, respectively, together with D-glucitol, whereas maltitol yields no 1,6-anhydride; the 1,6-anhydrides are extensively decomposed under the reaction conditions and hence do not accumulate. More interesting is the formation of 1,4-anhydro-D-glucitol from all three disaccharide-itol and from D-glucitol itself. The mechanism leading to this product is unknown. The alkali-sensitivity of the alkyl glycosides is enhanced by a C₂-hydroxyl group *trans*-related to the glycosidic substituent (144), and steric effects analogous to those encountered in the acidic hydrolysis of O-glycosides (145) appear to be operative. However, since glycosides in which the C₂-hydroxyl group is *cis* to the glycosidic substituent are degraded by alkali, a mechanism not involving a 1,2-epoxide must occur (possibly nucleophilic displacement of the phenoxide group by hydroxyl ion). The alkali-lability is also influenced by the nature of the glycosidic alkoxy group (146), and the lability sequence *t*-butyl > *i*-propyl > ethyl > methyl has been observed for β -D-glucopyranosides. The above reactions were performed in the absence of oxygen. Zienius & Purves (147) observed that the methyl ester of methyl-D-galacturonoside was converted to methyl-D-galacturonoside by aqueous sodium hydroxide in the absence of oxygen and to D-galacturonic acid in its presence.

Although not yet used for this purpose, oxoglycosides (page 49) should be valuable for the synthesis of branched-chain sugars, many of which are naturally occurring. The 5-oxo derivatives obtained from methyl 2,3-O-isopropylidene-L-rhamnofuranoside (72) on reaction with methyl magnesium iodide followed by acidic hydrolysis yield noviose (5-deoxy-5-C-methyl-L-lyxohexose). Aldoses, substituted to preclude ketose and enediol formation, undergo normal aldol condensations under mild alkaline conditions. Thus, 5-aldo-1,2-O-isopropylidene- α -D-xylopentofuranose (obtained by glycol cleavage of 1,2-O-isopropylidene-D-glucofuranose) yielded a derivative of a 9-aldo-4-C-formylnonose (148), and 2,4-O-ethylidene-D-erythrose afforded a derivative of a 3-C-formylheptitol (149). Likewise, 5-aldo-1,2-O-isopropylidene- α -D-xylopentofuranose reacted with formaldehyde, but the product then underwent a crossed Cannizaro reaction, and after acidic hydrolysis 4-C-hydroxymethyl-L-threopentose was obtained (150). Oxidation to the pentonic acid and Ruff degradation gave L-apiose (3-C-hydroxymethyl-L-glycerotetrose). L-APIose has also been synthesised from (+)-tartaric acid (151) and from 3-O-benzyl-D-fructose via the cyanohydrin adduct (152). 2,3:4,5-Di-O-isopropylidene-aldehydo-D-arabinose condenses with compounds containing active methylene groups to give branched-chain sugar derivatives (153). This and other interesting results have arisen from the extensive study by Zinner and co-workers on sugar dithioacetals (154).

The aldol condensation of dihydroxyacetone and glycolaldehyde is catalysed (155) by amino acids at pH 6.5.

HYDROGEN BONDING

Although little is known about the precise manner in which an enzyme becomes attached to its substrate prior to bringing about a transformation, hydrogen bonding may be involved at least in some cases. The effect of hydrogen bonding on the properties of certain macromolecules is well-known; the shape of certain peptides (156) and proteins may be controlled and the insolubility of cellulose in water determined by hydrogen bonding. Intramolecular hydrogen bonds are a significant feature of the Watson-Crick model of deoxyribonucleic acid. Bruce *et al.* (157) have shown that certain carbohydrates, such as inositol, but not simple sugars, form complexes with proteins in solution. The phosphorylating ability of ATP *in vivo* may be attributable (158) to enhancement of activity by hydrogen bonding with the enzyme protein, since ATP is a weak phosphorylating agent *in vitro*. Uridine-5'-phosphate forms an inclusion compound by hydrogen bonding in aqueous solution with β -Schardinger dextrin. Even when the solution is buffered at pH 7, the ultraviolet absorption spectrum of the included nucleotide indicates an alkaline environment (158). Thus, patterns of hydrogen bonding may affect local control of pH. Inclusion compounds of cyclic dextrans have been studied in detail (159).

Formation of a hydrogen-bonded complex is suggested to explain the efficiency of 2-aminopyridine as a catalyst in the hydrolysis of certain esters (160). Such reactions may serve as models for esterase activity (161). Thus, it is possible that intermolecular hydrogen bonding assists in the formation of an enzyme-substrate complex and that, within the complex, intramolecular hydrogen bonding helps activate the substrate to bring about a specific reaction. A variety of functional groups, present in enzyme proteins, may act as proton acceptors or donors in hydrogen bonding. The hydrogen-bonding tendencies of various functional groups are known (162), and the nature of the hydrogen bond has been discussed in detail (163, 164).

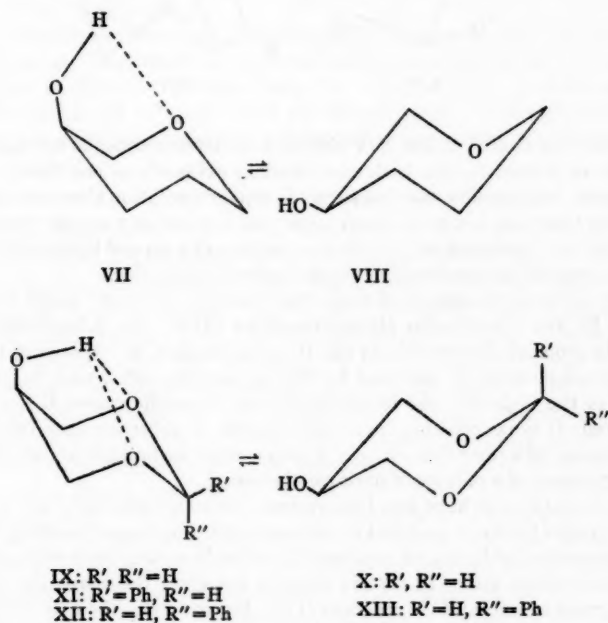
Information on possibilities for hydrogen bonding in carbohydrates is obtained from simple diols by infrared spectroscopy on solutions of the compounds in carbon tetrachloride (165). The extent of intermolecular hydrogen bonding is controlled by the concentration, and in very dilute solutions only intramolecular hydrogen bonding occurs. Examination of hydrogen-bonding patterns in hydroxy compounds is limited to those alcohols that are soluble in solvents the molecules of which do not themselves exhibit significant hydrogen bonding, for example, the paraffins and carbon tetrachloride. Sugars are normally insoluble in these solvents, and hydrogen-bonding patterns cannot easily be studied directly. The effects of hydrogen bonding on reaction rates may be assessed from simple molecules, which are also amenable to spectroscopic study, and the converse approach, viz., the analysis of

hydrogen-bonding effects by examination of reaction rates and patterns, appears feasible in application to more complex compounds. Kuhn's method has been widely applied to alcohols and amino alcohols (166).

Relatively little is known of hydrogen bonding involving thiols and sulphides. In acyclic diols, where relatively free rotation about bonds can occur, five-, six-, and seven-membered rings, but not larger ones, may be formed by intramolecular hydrogen bonding. Rings larger than seven-membered might result from restricted rotation. Intramolecular hydrogen bonding in carbocyclic vicinal diols is influenced in the anticipated manner by steric effects. For a particular compound, the magnitude of the arithmetical difference ($\Delta\nu$) between the frequencies (ν) of free and bonded hydroxyl groups is regarded as an index of strength and, hence, length of a hydrogen bond. The $\Delta\nu$ values associated with five-, six-, and seven-membered rings formed by intramolecular hydrogen bonding are quite different. However, there are no cases reported yet in which, within a molecule, there is a competition of hydrogen bonds involving the formation of intramolecular rings of different sizes. The tendency of hydroxyl groups to form hydrogen bonds should normally parallel their acidities and follow the sequence tertiary < secondary < primary. This sequence may be altered in certain cases by steric effects. Thus, in cyclohexane vicinal-*cis* diols, the axial hydroxyl group, whether secondary or tertiary, is crowded by axial hydrogen atoms on the same side of the ring and oriented such that its oxygen atom is not accessible for hydrogen bonding with the neighbouring equatorial hydroxyl group (167). The energy of intermolecular hydrogen bonds between secondary hydroxyl groups and ether oxygen atoms (168) is approximately 3.2 kcal/mole. It is likely that the energy of intramolecular hydrogen bonds will be of a similar magnitude. The involvement of the ring oxygen atom in derivatives of tetrahydropyran and tetrahydrofuran in intramolecular hydrogen bonding with suitably located hydroxyl groups has recently been recognised (169). For example, in carbon tetrachloride, tetrahydropyran-3-ol exists (170) as an equilibrium mixture of approximately equal amounts of the conformations VII and VIII.^a The intramolecular hydrogen bonding between the axial hydroxyl group and the ring oxygen atom in Formula VII stabilises the conformation, since in cyclohexanol approximately 70 per cent of the hydroxyl groups are in equatorial positions (171). The C₂-hydroxyl group in mannopyranosides and the C₄-hydroxyl group in galactopyranosides (both in the C1 conformation) are sterically located, as in Formula VII, and may be hydrogen-bonded to the ring oxygen atom. When two-ring oxygen atoms are present, as in 1,3-*O*-methyleneglyceritol (1,3-dioxan-5-ol), the molecule exists predominantly in conformation IX. Configurations have been allo-

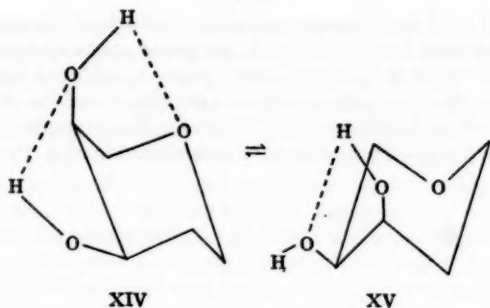
^a For the purpose of simplicity, it is assumed that the six-membered ring compounds considered in this chapter exist in true chair conformations. However, non-bonded interactions in certain of the compounds may lead to deformation, the extent of which would be difficult to assess.

cated to the 1,3-*O*-benzylideneglyceritols on the basis of the extent of intramolecular hydrogen bonding. The *cis* compound exists exclusively in conformation XI (bonded hydroxyl only), which is anchored by the phenyl group, whereas the *trans*-isomer exists as a mixture of conformations XII and XIII; the latter predominates (172). This is the first example of the application of the method in carbohydrate structural determination. By this method,



the location (2 or 5 positions) of the tosyl group in the monotosyl derivatives of 1,4-3,6-dianhydro-D-glucitol has been shown to be the reverse of that previously believed (173). The hydrate of 2-phenyl-1,3-dioxan-5-one may be stabilised by hydrogen bonding.

Significant observations have also been made with certain tetrahydropyran diols (169). Thus, 1,5-anhydro-2-deoxy-L-erythropentitol exists in carbon tetrachloride as a mixture of the conformations XIV and XV; the latter predominates since it contains two intramolecular hydrogen bonds. The molecular rotations (-45° and $+88^\circ$, respectively) for the conformations XV and XIV may be calculated (174), and the observed rotation ($+75^\circ$)



indicates that conformation XIV also predominates in aqueous solution. The pattern of intramolecular hydrogen bonding evidently is not disrupted by hydration. It is possible that for sugars in aqueous solution there may be two layers of hydrogen bonds, an inner layer which involves a specific pattern or cumulation of intramolecular hydrogen bonds and a second layer which comprises a cage of intermolecularly bonded solvent molecules.

The influence of change of shape on chemical reactivity might be illustrated by the 3,6-anhydro glycopyranosides (175). The 3,6-anhydro ring fixes the molecule irreversibly in the 1C conformation, as opposed to the C1 conformation normally assumed by the glycopyranosides, and, because of strain in the molecule, the reactivity of the glycosidic centre is markedly enhanced. It is conceivable that enhancement of substrate reactivity as a consequence of a reversible change of shape might be brought about enzymically by means of a pattern of hydrogen bonding.

Several examples have now been recorded in which the reactivity of functional groups has been modified by intramolecular hydrogen bonding. Thus, the intramolecular hydrogen bonding of a suitably located hydroxyl group to an axial acetoxy group in certain steroids enhances the sensitivity of the latter group to nucleophilic reagents (176). For example, the rate of saponification exceeds that of an unbonded equatorial acetoxy group; the reverse order of reactivity is the case with unbonded groups. The unexpected selective tosylation of 1,4-3,6-dianhydro-D-glucitol at the apparently sterically inaccessible C₄-hydroxy group rather than at the apparently exposed C₂-hydroxyl group is probably caused by hydrogen bonding. The C₆-hydroxyl group is completely bonded to the C₁-oxygen atom, whereas the C₂-hydroxyl group is unbonded (169). Involvement of a hydroxyl group in hydrogen bonding of the type $\text{—O—H} \cdots \text{X}$ would tend to increase the basicity of the oxygen atom and thereby enhance the reactivity of the hydroxyl group toward electrophilic reagents. Likewise, the reactivity of the hydroxyl group in *cis*-1,3-*O*-benzylidene glycerol is enhanced (177). Hydrogen-bonding effects have been invoked to explain the reaction of D-arabitol with benzaldehyde to yield a 1,3- in preference to a 3,5-*O*-benzylidene derivative (177).

The operation of a similar effect should direct the reaction of β -sedoheptitol (D-glycero-D-glucoseptitol) to yield a 1,3-2,4-5,7- rather than a 1,3-4,6-5,7-tri-*O*-benzylidene derivative. The reaction of glycerol with benzaldehyde differs from that of the higher polyhydric alcohols in that it yields, under the usual reaction conditions, predominantly five-membered cyclic acetals, intermolecular, as distinct from intramolecular, hydrogen bonding probably controls the reaction pattern (178), since in very dilute solution, where intermolecular hydrogen bonding is precluded, the six-membered cyclic acetal is the main product.

Intramolecular hydrogen bonding (179) appears to control the stereospecificity of the epoxidation of glycals and related compounds, the pattern of which is markedly different from that observed for the epoxidation of acetylated glycals (179). Most of the above reactions take place in polar solvents so that intramolecular hydrogen-bonding effects are not swamped by solvation.

CYCLIC ACETALS

D-Mannitol reacts with 1,1,1-trifluoroacetone to yield, *inter alia*, a tri-ketal and two 1,2-5,6-diketals, which are isomeric at the ketal carbon atoms (180). The ketals are extremely acid-resistant but are broken down by boron trichloride, a versatile reagent which will also readily demethylate carbohydrate methyl ethers (181). Boron trichloride and boron tribromide also effect deacylation and the degradation of di- and polysaccharides to monosaccharides. Hexoses are largely unaffected by the reagent, but ketoses are converted to 5-hydroxymethylfurfuraldehyde. Methyl ethers may be used as blocking groups in carbohydrate synthesis and subsequently be removed by boron trichloride (65). The mechanism of the reaction of boron trichloride with cyclic acetals has been studied in detail (182). A novel hydroxylated linear polyester (mol. wt. ca. 5000) has been obtained (Figure 6) by treating 1,3:2,4:5,6-tri-*O*-methylene-D-glucitol with adipic acid and trifluoroacetic anhydride (183). Cross-linked polyesters were obtained unexpectedly when di-*O*-methylenepentaerythritol was similarly treated (184). Acyl trifluoroacetates (prepared from equimolar quantities of carboxylic acids and tri-

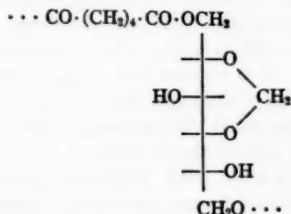


FIG. 6. Polymer from action of trifluoroacetic anhydride and adipic acid on 1,2:3,4:5,6-tri-*O*-methylene-D-glucitol.

fluoroacetic anhydride) cleave (185) methylene derivatives of D-glucitol, which contain α - or β -rings, to yield, after mild hydrolysis, compounds with free secondary hydroxyl groups; β T but not β C rings are also cleaved (186). A new approach to partially substituted polyhydroxyl compounds is thereby provided. The 5,6-bistrifluoroacetate of 1,3:2,4-di-O-ethylidene-D-glucitol may be converted variously to 5- and 6-substituted derivatives in some cases by routes that involve acyl migration (187). Selective protection of sugar hydroxyl groups may also be effected by conversion to carbonates (188). In the presence of aqueous alkali, aryl chloroformates react with sugars to yield O-aryloxycarbonyl derivatives and, with favourable steric conditions, cyclic carbonates. The former derivatives only may be cleaved by hydrogenolysis.

A novel cyclic acetal migration occurs when 2,4-O-ethylidene-D-erythrose is treated with methanolic hydrogen chloride (189). Methyl 2,3-O-ethylidene-D-erythrofuranoside is formed; it contains the stereochemically favourable arrangement of two five-membered rings in *cis* fusion. The oxidation and reduction products of 2,4-O-ethylidene-D-erythrose have been examined (190). A seven-membered ring compound, *trans*-hexahydro-1,3,5-benzotrioxepan, is formed, *inter alia*, when cyclohexan-*trans*-1,2-diol reacts with formaldehyde (191). The *cis* diol yields the normal methylene compound. Seven-membered rings are formed in the reactions of methyl α -D-glucopyranoside with acetaldehyde and rhamnose with formaldehyde. D-Mannose yields a 2,3:5,6-di-O-cyclohexylidene derivative with cyclohexanone (192), and D-glucurone dialkylthioacetals yield 2,4-benzylidene and ethylidene derivatives (193).

Ethyl metaphosphoric acid is a convenient catalyst for the preparation of carbohydrate isopropylidene derivatives (194). Isopropylidene residues are usually stable under mild alkaline conditions but may be labilised by certain vicinal groups; thus, the 3,5-cyclic ketal residue in 1,2:3,5-di-O-isopropylidene-6-nitro-6-deoxy-D-glucofuranose is alkali-labile, whereas that in the corresponding L-idose derivative is stable (195). High pressure hydrogenation (196) of 1,2-O-isopropylidene-D-glucofuranose at 180° over copper chromite gave a mixture of variously hydroxylated hexanes together with 1,2-O-isopropylidene-L-idofuranose. Numerous other sugar derivatives have been subjected to vigorous hydrogenation (197). Under more drastic conditions, the 5,6-ketal ring in 1,2:5,6-diketals of D-glucose is selectively cleaved to yield 6-O-alkyl derivatives of D-glucose and L-idose; isomerization at C₄ probably occurs after cleavage of the ketal ring (198). L-Iditol affords (199) a 1,3:2, 4:5,6-tri-O-benzylidene derivative and a di-O-benzylidene derivative which probably (200) has a 2,4:3,5-distribution of the cyclic acetal groups.

The reaction of benzaldehyde with polyhydric alcohols to yield six-membered cyclic acetals creates a new asymmetric centre and hence, the formation of diastereoisomers is possible. Mills (201) suggests that only the diastereoisomer with the substituent at the aldehyde carbon atom equatorial to the 1,3-dioxan ring will, in fact, be formed. Compounds considered to be pairs of diastereoisomers have been shown to be dimorphs (202).

A scheme for the degradation of D-glucose so that each carbon atom may be separately isolated as carbon dioxide starts from 4,6-O-ethylidene-D-glucose (203). Xylose diethyl thioacetal yields a 2,4:3,5- not a 2,3:4,5-di-O-benzylidene derivative (204).

CARBOHYDRATE SULPHATES

Ester sulphate residues are present in many polysaccharides, and there is need of a general method for proving the molecular location of these groups; the *trans* esterification method (205) does not work in every case. For example, heparin with five sulphate groups per tetrasaccharide unit is only partially desulphated with methanolic hydrogen chloride (206) and loses three sulphate groups (two O- and one N-); the remaining N-sulphate is removed on mild acid hydrolysis, leaving a heparin with one sulphate group per unit. Polysaccharides containing sulphated primary hydroxyl groups (chondroitin sulphate C) or axial secondary hydroxyl groups (chondroitin sulphates A and B) are desulphated by methanolic hydrogen chloride. The response of sulphated equatorial secondary hydroxyl groups to the reagent is unknown. Sulphate groups may be removed from simple carbohydrates with lithium aluminium hydride (207), but solubility problems complicate application of the reagent to polysaccharides. The location of sulphate groups may be correlated with the nature of the products arising on basic hydrolysis. Thus, a variety of hexoses are produced when dextran sulphate is treated with alkali and then hydrolysed with acid (208). Epoxide formation, migration, and cleavage account for the sugars formed (209). Cyclic sulphates are not intermediates (210); they are formed in the reaction between glycosides and sulphuryl chloride (211).

An alternative approach is the fragmentation of sulphated polysaccharides to oligosaccharides in which the sulphate groups are retained. Painter (212) has observed that the yield of oligosaccharides in an enzymic degradation is improved if they are continually removed by dialysis. The same principle was applied to the autohydrolysis of the polysaccharide from the red algae, *Furcellaria fastigiata* (which contains D-galactose, 3,6-anhydro-D-galactose and sulphate moieties), and it was found that 60 per cent of the polysaccharide became dialysable but only 20 per cent of the ester sulphate groups were hydrolysed (213). The procedure has been further improved by the use of a water-soluble, non-dialysable (i.e., polymeric) acid to bring about the hydrolysis (214).

Chlorosulphonic acid has been used in an improved synthesis of hexose- and hexosamine-6-sulphates (215). Other workers prefer the pyridine-sulphur trioxide complex as the sulphating agent (216). The sugar sulphates may be chromatographed either as salts or as the free acids. Chromatographic mobilities are increased by the incorporation of cationic detergents into the solvent system (217). Glucose-6-sulphate is the main product of the reaction (218) of glucose with bisulphite at 100°.

Lloyd & Dodgson have summarised (219) evidence which shows that, whereas hexose-6-sulphates have infrared absorption at 820 cm.⁻¹, carbo-

hydrates that contain galactose-4-sulphate moieties absorb at 850 cm^{-1} . A C-4-sulphate group in galactose is probably axially disposed, but it is not yet apparent whether the absorption at 850 cm^{-1} is generally indicative of axial sulphate groups.

MISCELLANEOUS

The preparation of aldoses labelled with tritium at the glycosidic centre has been described (220). Weygand *et al.* (221) have found that, on fractional crystallization of mixtures of mannose-1- ^3H -phenylhydrazone and its glucose analogue, some isomerization occurs.

Several examples have now been reported of the direct inversion of configuration of individual carbon atoms in carbohydrates without the involvement of epoxide formation or neighbouring group reactions. Such reactions are of potential value for the synthesis of rare sugars. Treatment of 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-mesyl- β -D-galactose with potassium fluoride in methanol gave 1,6-anhydro-3,4-*O*-isopropylidene-2-*O*-methyl- β -D-talose (222). Sodium benzoate in dimethyl-formamide converts (223) methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-tosyl- α -D-galactopyranoside into methyl 2,3,4,6-tetra-*O*-benzoyl- α -D-glucopyranoside. This reagent will undoubtedly find wide application.

Recent years have seen the application of the principles of conformational analysis to a variety of carbohydrate reactions (20). Various conformations of pyranose rings have been invoked as reaction intermediates, and clearly some system of nomenclature is necessary for convenience in discussion. Reeves' original system covered only boat and chair conformations, but, more recently, this author has described a series of geometrically depictable conformations of pyranose rings, and these provide a logical basis for a nomenclature system. The naming of various conformations has been attempted (224, 225), but incomplete nomenclature systems are unlikely to gain general acceptance. An internationally agreed system should be devised as soon as possible and thereafter used exclusively. From the extent of complex formation with cuprammonium, Bentley has deduced that, whereas α -idopyranosides exist in the 1C conformation, β -idopyranosides probably adopt a half-chair conformation (226). The same author (227) concludes that both the glucose units in cellobiose adopt the $\text{C}1$ conformation, whereas with maltose the reducing unit adopts the $\text{C}1$ conformation but the non-reducing component is probably in a skew conformation. The conformation assumed by amylose in aqueous solution is that of an unperturbed random coil (228).

The final proof of structure of oligo- and polysaccharides must be based on the conversion to known crystalline derivatives. Methylation is still widely applied in this respect, although graded acidic hydrolysis and fragment identification is being used increasingly. Recognition (229) that the methylation of alcohols by diazomethane is strongly catalysed by fluoroboric acid will undoubtedly have repercussions in the carbohydrate field.

O-Methylation of polysaccharides that contain *N*-acetylated amino sugar moieties can be effected by the Haworth method (dimethyl sulphate/sodium hydroxide), whereas use of the Freudenberg method (sodium/methyl iodide/liquid ammonia) leads also to *N*-methylation (230). Considerable attention is still focused on the syntheses of new reference methylated sugars and on improving older synthetic methods. The synthesis of 5-*O*-methyl-L-arabinose (231) and 2,3-di-*O*-methyl-D-arabinose (232) has been reported, as has an alternative synthesis of 2-*O*-methyl-D-xylose (233). 1→2-Acyl migration occurs on methylation of the 1,3,4,6-tetra-*O*-acetyl-D-glucoses (234).

Partial methylation (235) of xylan (from esparto grass) with dimethyl sulphate in sodium hydroxide showed the C₂-hydroxyl to be more rapidly substituted than the C₃-hydroxyl group. In cellulose, the C₂- and C₆-hydroxyl groups are the most reactive (236). However, methylation of cellulose with diazomethane and methyl 4,6-*O*-ethylidene-β-D-glucopyranoside with dimethyl sulphate and sodium hydroxide revealed no marked difference in reactivity of the various hydroxyl groups (237). A variety of methyl ethers are formed when the crystalline trisodium derivative of sucrose is treated with methyl toluene-*p*-sulphonate (238); the most reactive hydroxyl groups are those at C₂ in the D-glucose moiety and at C₁ in the D-fructose moiety. Methylation of D-glucose diethyl thioacetal under homogeneous conditions reveals that the C₂-hydroxyl group is the most reactive (239).

Partial esterification of sugars may be accomplished by using limited amounts of reagent (1). The converse approach has now been described. Graded saponification of penta-*O*-acetyl-D-glucose yields D-glucose 6-acetate (240).

The nature of the products arising from the action of ionizing radiation on carbohydrates is dependent on the presence or absence of oxygen. Thus, irradiation of an aqueous solution of glucose with ultraviolet light and in the presence of oxygen yields arabinose as the main product (241). Under similar conditions, γ-ray irradiation of mannose (242) yields a variety of products, including mannonic and mannuronic acids, arabinose, and erythrose. γ-Ray irradiation of aqueous solutions of D-glucose in the absence of oxygen yields an unusual acid- and alkali-stable polymer, probably formed by way of D-gluconic acid and a free radical polymerization (243). The stability may be due to the linkage of the sugar moieties by carbon-carbon bonds. 2-Oxo-derivatives of D-glucose are formed in addition to the polymer (244). A variety of simple carbohydrates yield polymers on irradiation; polymer formation is inhibited by oxygen. Irradiation of glycolic acid yields the radical ·CH(OH)·CO₂H, which then reacts to yield a variety of carboxylic acids (245).

Carbohydrates in which hydroxyl groups have been replaced by fluorine atoms may be of value as metabolic blocking agents and in mechanistic studies. 1,2,3,4-Di-*O*-isopropylidene-6-*O*-mesyl-D-galactose is converted to the 6-deoxy-6-fluoro derivative on treatment with anhydrous potassium fluoride in ethylene glycol (246). 6-Deoxy-6-fluoro-D-galactose was sub-

sequently obtained crystalline, and its properties examined in detail (247). 5-Deoxy-5-fluoro-D-ribose was obtained by a similar route. Oxidation of (\pm)-2-deoxy-2-fluoroglyceraldehyde (248) with hypohalite yields (\pm)-2-deoxy-2-fluoro-glyceric acid (249), which was resolved as the quinine salt. 2-Trifluoromethylglycerol has been synthesised from 3-bromo-1,1,1-trifluoropropan-2-one (250).

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CHEMISTRY OF LIPIDS¹

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The new *Journal of Lipid Research* (1) is a welcome addition to scientific literature. It contains not only original papers on all aspects of lipid chemistry, biochemistry, and physiology but also valuable review articles, and the editorial staff prepares "Notes on Methodology" and a section on "New Methods," which contains a comprehensive list of new methods.

Gunstone (2) has published an *Introduction to the Chemistry of Fats and Fatty Acids*, and a two-volume treatise on *Lipid Chemistry* and *Lipid Metabolism*, edited by Hanahan *et al.* (3) and Bloch (4), respectively, has just been completed.

As usual, the reviewers had to choose selected topics only because of the limited space available. Analytical methods and new chemical structures are treated in detail; sterols and steroids are excluded from this review.

METHODS OF ANALYSIS

FATTY ACID CHROMATOGRAPHY

Recent reviews on the chromatography of lipids include those of Asselineau (5) and Michalec & Sobeslavsky (6); Asselineau (7) has also published a review on the chromatography of fatty acids.

Column chromatography.—Partition chromatography of 4-chlorophenacyl esters of fatty acids on a column of polyethylene-celite gives good resolution from C₁₀ to C₁₈ (8). Reversed-phase chromatography on rubber powder of fatty acids from C₁₁ to C₁₈ has been studied (9). Silicone grease on Celite is a convenient stationary phase; odd-numbered straight-chain fatty acids have been separated by this procedure, the mobile phase being aqueous acetonitrile (10).

Paper chromatography.—Fats can be directly saponified on a paper sheet in view of the subsequent chromatographic analysis of the liberated fatty acids (11). Long-chain fatty acids are best separated by using paraffin oil as stationary phase and aqueous acetone or acetic acid as mobile phase. A simplified procedure for routine work has been published by Chayen & Linday (12). Homologous α -hydroxy, α -methyl fatty acids have been separated by a similar method (13). Separation of saturated acids from C₂₀ to C₂₄ has been obtained by running the chromatography at 85°C. (14). Fatty acids from C₁₄ to C₂₄ from brain sphingolipids have been studied by the paper-chromatographic system of paraffin oil/95 per cent acetic acid, in which acid spots are detected as bismuth sulfides (15). Polyunsaturated acids have been identified and quantitatively estimated (16). To avoid interference of unsaturated

¹ The survey of the literature pertaining to this review was concluded in September, 1960.

acids with saturated ones, the former can be hydroxylated by developing the chromatogram by a mixture (6:1:1) of acetic acid, formic acid, and hydrogen peroxide (17). Unsaturated acids have been separated as polybromo derivatives on silicic acid-coated paper (18). Higher fatty acids from plant tissues have been analyzed by chromatography on rubber-coated paper (19). Fatty acids from C_8 to C_{11} have all been separated by using 2-ethyl-1-hexanol saturated with aqueous ammonia as the mobile phase (20).

Vapor-phase chromatography.—An excellent review on "The Qualitative and Quantitative Determination of the Fatty Acids by Gas-Liquid Chromatography" has been written by James (21; see also 21a). Silicone grease is a very versatile stationary phase, but it gives practically no separation between saturated and unsaturated fatty esters of the same carbon number (see 22); Apiezon L is somewhat better in this respect (23). Use of polyesters of dibasic acids and glycols (e.g., the commercial polymer Reoplex 400) as a stationary phase gives excellent separation according to the number of double bonds, the retention time increasing with the degree of unsaturation (24 to 28). Such phases can be stabilized by complete elimination (by passage on a weak base-exchanger) of the acids that have been used for their preparation (29). Polyvinyl acetate may also be used (30). However, decomposition of dimethyl malonate on such a type of stationary phase has been observed, whereas no decomposition occurs on silicone grease (30a). α -Hydroxy esters prepared from brain cerebrosides have been separated by gas-liquid chromatography by using either their methyl ester (31) or the C_{n-1} fatty acid arising from oxidative degradation (31a).

The efficiency of columns has been greatly improved by using Golay-type capillary columns. The maximum amount of a single ester that can be applied on such a capillary column is of the order of 0.1 μ g. Efficiencies up to 200,000 theoretical plates have been obtained (32).

The chromatographic behavior of methyl esters of normal odd-numbered, *iso*, and *ante-iso* fatty acids from C_8 to C_{30} has been studied (33). Methyl esters of fatty acids up to 34 carbon atoms have been separated on a silicone-grease column, at 295° (34), and those of branched-chain fatty acids (from tubercle bacillus), up to 34 carbon atoms (35). Samples of pure esters can be collected at the end of the column and used for infrared spectrography or mass spectrometry (see 35).

A linear relationship between chain length and logarithm of retention time has been observed (24, 35, 36, 37); parallel straight lines are obtained for various homologous series of methyl esters of fatty acids. Such representation may be of help in the identification of an unknown ester.

Considerable decomposition of methyl *cis*-9,10-epoxyoctadecanoate on a column of silicon grease at 235° can be eliminated by using Celite as the solid support instead of C-22 firebrick (38). Stoffel *et al.* (25) have observed no decomposition of highly unsaturated C_{16} to C_{18} fatty acid methyl esters on columns of Apiezon M (a thermostable non-polar paraffin grease) or Reoplex 400 at 197°.

Other.—Fatty alcohols have been separated by paper chromatography (39,

40) or by vapor-phase chromatography (41). Ion-exchange chromatography has been used for wax analysis (42). Aliphatic components of hydrolyzed wool wax have been identified by their transformation into alcohols (LiAlH_4 reduction), chromatographic separation of the monohydroxy-, α,β -dihydroxy- and α,ω -dihydroxy-alkanes, and reduction of each group to hydrocarbons that have been analyzed by gas chromatography (42a).

LIPID CHROMATOGRAPHY

Lipids can be detected on paper chromatograms by iodine (43) or by an acid solution of protoporphyrin (44). The content of lipid compounds that are soluble in pure methanol and insoluble in a methanol-water (1:2) mixture can be determined by nephelometry in column effluents (45). Free fatty acids are analyzed by adsorption on an anionic exchanger, transformation *in situ* into methyl esters, and vapor-phase chromatography of the esters (46).

Silica-impregnated paper has been used for the analysis of glyceride mixtures: triglycerides are easily separated from mono- and diglycerides (47, 48), but further fractionation can be achieved only in very particular cases. Transformation of monoglycerides into fatty esters of allyl alcohol by treatment with $\text{CH}_2=\text{SO}_2\text{Cl}$ allows their separation by gas-liquid chromatography (49).

The phospholipids of rabbit skin (50), alcohol-soluble phospholipids of soy bean (51), and phospholipids of wheat (51a) have been studied by column chromatography on silicic acid or cellulose powder (see also 52, 53). A concave concentration gradient of methanol in chloroform, employed for the elution of lipids from a silicic acid column, gives a better resolution of complex mixtures (54). Marinetti, Stotz, and others (55, 55a, 55b, 56) have continued their work on the analysis of phospholipids by paper chromatography. Octanol-lutidine-acetic acid (45:2.5:5) and 2,6-dimethyl-4-heptanone-acetic acid (30:5) are useful solvents for development. Phosphatides and sphingomyelin have been separated on glass paper coated with sodium silicate; such a glass paper appears to be superior to one coated with silicic acid because of the simplicity of its preparation and the ease with which glass papers with the same properties can be constructed (57). Diphosphatidyl-glycerol (cardiolipin) has been separated on silicic acid-impregnated paper and estimated by neutron activation (58). Perrin & Saunders (59) have reported experiments on the fractionation of phosphatides by ion-exchange chromatography. Baker & Porcellati (60) have described the separation of nitrogen-containing phosphate esters from brain and spinal cord by ion-exchange chromatography.

Sphingomyelins and sphingosine have been purified by chromatography on silica (61). Periodic acid oxidation of sphingosine yields palmitaldehyde, which can be determined by vapor-phase chromatography (62); this method has been used for the investigation of the sphingomyelin fraction of human plasma lipids (63).

Two methods for the detection of cerebrosides on paper chromatograms

have been devised; a combination of the two gives a specific identification (64). The fatty acid components of cerebrosides have been investigated by gas-liquid chromatography (31).

COUNTERCURRENT DISTRIBUTION

Acetonitrile-pentane is a convenient solvent system because of its selectivity toward unsaturation; the low boiling point of these solvents allows an easy recovery of the esters of fatty acids. This procedure has been applied to the purification of methyl linolenate (65), arachidonate (65), and methyl dimorphenolate (66).

Isolation of a dihydroxy acid by means of countercurrent distribution between petroleum ether and 85 per cent ethanol has been achieved. Countercurrent distribution has been used for the study of the glyceride composition of cocoa butter (68), the separation of dinitrophenylated and methylated phospholipids (69), and the analysis of mixtures of complex lipids (52, 69a).

INFRARED SPECTROGRAPHY

Quantitative estimation of *trans*-unsaturated C_{18} fatty acids has been investigated (70). The position of the double bond in *trans*-6- through -11-octadecenoic acids can be determined by studying the absorption bands in the 1180 to 1350 cm^{-1} region (71), which is related to the length of the chain segment between the double bond and the carboxyl group.

The amount of lecithins and sphinomyelins contained in a complex mixture of lipids can be determined by infrared absorption measurements of fractions obtained by chromatography (on silicic acid) (72). Infrared spectra of polymorphic forms of glycerides have been studied (73).

NUCLEAR MAGNETIC RESONANCE SPECTROGRAPHY

The presence of a cyclopropane ring in sterculic acid has been definitely established by nuclear magnetic resonance spectrography (74). This method has been used in the course of a study of a phosphoryl ethanolamine derivative of batyl alcohol isolated from egg yolk (75). The nuclear magnetic resonance spectrum of methyl arachidonate has been described by Privett *et al.* (76). Only few other applications have been made in the lipid field (77 to 79).

MASS SPECTROMETRY

A combination of vapor-phase chromatography and mass spectrometry affords an extremely powerful tool for the investigation of complex mixtures of fatty acids (see 35, 80). Behavior of straight-chain fatty acid esters has been thoroughly studied by Ryhage & Stenhagen (81). Considerable information can be obtained on the structure of branched-chain esters (35, 82, 83, 84), of unsaturated esters (85), of esters that contain an oxygenated function (86), or of higher branched-chain alcohols (87).

MISCELLANEOUS METHODS

Complex mixtures of lipids have been fractionated by dialysis with wetted

cellulose sacs in a chloroform-methanol (2:1) medium (88). Ester groups in lipids have been determined by a simplified spectrophotometric method (89). Procedures for the identification and quantitative estimation of ethanolamine (90), serine (90), and inositol (91) in lipid hydrolyzates have been described.

Unsaturated fatty acids can be separated from saturated fatty acids on a preparative scale by means of their adducts with mercuri-acetate (92). Mercaptoacetic acid reacts quantitatively with the double bond of unsaturated fatty acids; this reaction has been used for the determination of tri-saturated glycerides in the presence of mixtures of unsaturated ones (93).

CHEMISTRY OF LIPIDS

FATTY ACIDS

New natural fatty acids.—Progress in the methods of analysis and fractionation has allowed the characterisation and isolation of new fatty acids, a list of which is given in Table I.

Three different C_{18} epoxyacids are now known; according to the location of the epoxy ring on the carbon chain, they may be biogenetically related to linoleic acids. Moreover, linoleic acid might be a biogenetic precursor of dimorphecolic acid (66) (see Table I, $C_{18}H_{32}O_2$), since 9-hydroxy-10,12- and 13-hydroxy-9,11-octadecadienoic acids are produced by autoxidation of linoleic acid. *Trans*-unsaturated acids might arise from hydrogenation processes of unsaturated acids by microorganisms in the rumen of cattle (94).

Twenty-three chemically defined unsaturated acids are known to occur in menhaden body oil (95). The presence of dienic and trienic non-conjugated C_{20} acids in tall oil obtained from south pine has been demonstrated (96). The *cis* form of 11-octadecenoic acid has been found in *Asclepias syriaca* seed oil (97). The toxic principle of *Dichapetalum toxicarium* has been isolated by Peters & Hall, who found it to be a fluorooctadecenoic acid (97a).

Branched-chain fatty acids have been characterized in the lipids of *Sporidesmium bakeri*, a fungus held responsible for the "facial eczema" disease affecting sheep and cattle (130). Several new branched-chain fatty acids have been isolated from bacterial lipids: sarcinic acid, $C_{15}H_{30}O_2$ (109); an acid $C_{17}H_{32}O_2$ containing a cyclopropane ring (113, 114); and nocardic acid, $C_{50}H_{96}O_2$ (129) (see Table I). The structure of the previously isolated mycocerosic acid (35) and mycolic acid, (131, 132) has been investigated. The biosynthesis of lactobacillic acid (optically active *cis*-11,12-methylene-octadecanoic acid) from *cis*-vaccenic acid (133, 134) and corynomycolic acid [(+)-2-tetradecyl-3-hydroxyoctadecanoic acid] from two molecules of palmitic acid (135) has been elucidated.

Stereochemistry.—Ricinoleic acid is 12(D)-hydroxyoctadec-*cis*-9-enoic acid, as shown by comparison of the 3-hydroxynonanoic and 12-hydroxy-octadecanoic acids prepared from the natural acid with the corresponding optically active synthetic compounds (136). A detailed report on the stereochemistry of phloionolic acid (racemic form of *threo*-9,10,18-trihydroxy-octadecanoic acid) has been published (137), and the stereochemistry of the

TABLE I
NEW NATURAL FATTY ACIDS

Empirical formula	Name	Formula*	Source	Reference
$C_9H_{16}O_2$	Drosophilin E	$HC\equiv C-C\equiv C-CH=CH-(CH_2)_6-COOH$	<i>Drosophila subrata</i>	98
$C_{10}H_{16}O_2$	Deca-2-en-4,6,8-trienoic	$CH_2-(C\equiv C)_3-CH=CH-COOH$	<i>Artemisia vulgaris</i>	99
		$CH_2-(C\equiv C)_3-CH=CH-COOH$	<i>Tripleurospermum bifontinus</i>	100
$C_{10}H_{16}O_2$	10-Hydroxydec-2-enoic	$HOCH_2-(CH_2)_8-CH=CH-COOH$	Royal jelly	101, 102
$C_{10}H_{16}O_2$	"Queen substance" (9-keto deca-2-enoic)	$CH_2-CO-(CH_2)_8-CH=CH-COOH$	<i>Apis mellifica</i> (Queen)	103 to 105
$C_{10}H_{16}O_2$	Deca- <i>trans</i> -2-en-4,6,8-triyn-10-oleic	$HOCH_2-(C\equiv C)_3-CH=CH-COOH$	<i>Ploutorus ulmarius</i>	106
$C_{10}H_{16}O_4$	Deca-2-en-4,6-diyn-1,10-dioic	$HOOC-(CH_2)_2-(C\equiv C)_2-CH=CH-COOH$	<i>Merulius lacrymans</i>	106
$C_{10}H_{16}O_4$	Deca-2,8-dien-4,6-diyn-1,10-dioic	$HOOC-CH=CH-(C\equiv C)_3-CH=CH-COOH$	<i>Polyporus guttatus</i>	106
$C_{11}H_{18}O_2$	Drosophilin C	$HC\equiv C-CH_2-(C\equiv C)_3-CH=CH-CH_2-COOH$	<i>Drosophila subrata</i>	98
$C_{11}H_{18}O_2$	Drosophilin D	$CH_2=C=CH-(C\equiv C)_2-CH=CH-CH_2-COOH$	<i>Drosophila subrata</i>	98
$C_{11}H_{18}O_2$	Nemotinic	$HC\equiv C-C\equiv C-CH=C-CH-CHOH-(CH_2)_2-COOH$	<i>Portia coriicola</i> , <i>Portia tenuis</i>	107
$C_{12}H_{20}O_2$	Dodeca-2,4-dienoic	$CH_2-(CH_2)_8-CH=CH-CH=CH-COOH$	<i>Sebastiano linguatrina</i>	108
$C_{12}H_{20}O_2$	Sarcinic	$CH_2-CH_2-CH(CH_2)-(CH_2)_{10}-COOH?$	<i>Sarcina lutea</i> , <i>Bacillus subtilis</i>	109
$C_{16}H_{30}O_2$	Hexadeca-8-enoic	$CH_2-(CH_2)_6-CH=CH-(CH_2)_8-COOH$	menhaden body oil	95
$C_{16}H_{30}O_2$	4,9-Hexadecadienoic	$CH_2-(CH_2)_8-CH=CH-(CH_2)_3-CH=CH-(CH_2)_2-COOH$	<i>Asclepias syriaca</i>	97
	6,9-Hexadecadienoic	$CH_2-(CH_2)_6-CH=CH-CH_2-CH=CH-(CH_2)_7-COOH$	menhaden body oil	95
			herring oil	110





* *c* and *t* designates the stereochemistry (*cis* or *trans*) of the double bond.

TABLE I—(Continued)

TABLE I—(Continued)

Empirical formula	Name	Formula*	Source	Reference
	7,10-Hexadecadienoic	$\text{CH}_2-(\text{CH}_2)_4-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_6-\text{COOH}$	herring oil	110
	9,12-Hexadecadienoic	$\text{CH}_2-(\text{CH}_2)_3-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{COOH}$	menhaden body oil	95
			herring oil	110
			<i>Asclepias syriaca</i>	97
$\text{C}_{16}\text{H}_{30}\text{O}_2$	4,7,10-Hexadecatrienoic	$\text{CH}_2-(\text{CH}_2)_4-(\text{CH}=\text{CH}-\text{CH}_2)_3-\text{CH}_2-\text{COOH}$	herring oil	110
	6,9,12-Hexadecatrienoic	$\text{CH}_2-(\text{CH}_2)_2-(\text{CH}=\text{CH}-\text{CH}_2)_3-\text{COOH}$	herring oil	110
	7,10,13-Hexadecatrienoic	$\text{CH}_2-\text{CH}_2-(\text{CH}=\text{CH}-\text{CH}_2)_3-(\text{CH}_2)_4-\text{COOH}$	menhaden body oil	95
	9,12,15-Hexadecatrienoic	$\text{CH}_2=\text{CH}-\text{CH}_2-(\text{CH}=\text{CH}-\text{CH}_2)_3-(\text{CH}_2)_6-\text{COOH}$	herring oil	110
$\text{C}_{16}\text{H}_{30}\text{O}_2$	4,7,10,13-Hexadecatetraenoic	$\text{CH}_2-\text{CH}_2-(\text{CH}=\text{CH}-\text{CH}_2)_4-\text{CH}_2-\text{COOH}$	<i>Senecioideus obliquus</i>	111
	6,9,12,15-Hexadecatetraenoic	$\text{CH}_2=\text{CH}-\text{CH}_2-(\text{CH}=\text{CH}-\text{CH}_2)_3-(\text{CH}_2)_3-\text{COOH}$	herring oil	110
$\text{C}_{16}\text{H}_{30}\text{O}_4$	10,16-dihydroxyhexadecanoic	$\text{HOCH}_2-(\text{CH}_2)_6-\text{CHOH}(\text{CH}_2)_6-\text{COOH}$	olive leaf oil	112
$\text{C}_{17}\text{H}_{32}\text{O}_2$		$\text{CH}_2-(\text{CH}_2)_n\text{CH}-\underset{\text{CH}_3}{\text{CH}}-(\text{CH}_2)_m-\text{COOH}$	<i>Escherichia coli</i>	113
			<i>Pasteurella pestis</i>	114
$\text{C}_{18}\text{H}_{34}\text{O}_2$	5-, 6-, 7-, 10-, 11-, and 12-Octadecenoic acids	$\text{CH}_2-(\text{CH}_2)_n-\text{CH}=\text{CH}-(\text{CH}_2)_m-\text{COOH}$	human faecal lipids	115
$\text{C}_{18}\text{H}_{34}\text{O}_2$	Octadeca-6,9-dienoic	$\text{CH}_2-(\text{CH}_2)_6-(\text{CH}_2-\text{CH}=\text{CH})_2-(\text{CH}_2)_4-\text{COOH}$	menhaden body oil	95
$\text{C}_{18}\text{H}_{34}\text{O}_2$	6,9,12-Octadecatrienoic acid	$\text{CH}_2-(\text{CH}_2)_4-(\text{CH}=\text{CH}-\text{CH}_2)_3-(\text{CH}_2)_4-\text{COOH}$	menhaden body oil	95
	<i>trans</i> -8,10,12-Octadecatrienoic acid	$\text{CH}_2-(\text{CH}_2)_4-(\text{CH}=\text{CH})_3-(\text{CH}_2)_6-\text{COOH}$	<i>Calendula officinalis</i>	116
$\text{C}_{18}\text{H}_{32}\text{O}_2$	Octadeca- <i>trans</i> -13-en-9,11-diynoic	$\text{CH}_2-(\text{CH}_2)_3-\text{CH}=\text{CH}-(\text{C}\equiv\text{C})_2-(\text{CH}_2)_7-\text{COOH}$	<i>Esocarpus cupressiformis</i> , <i>Exocarpus stritius</i>	117
$\text{C}_{18}\text{H}_{36}\text{O}_4$	2-Hydroxyoctadecanoic	$\text{CH}_2-(\text{CH}_2)_{15}-\text{CHOH}-\text{COOH}$	brain lipids	118

TABLE I—(Continued)

Empirical formula	Name	Formula*	Sources	Reference
$C_{18}H_{34}O_3$	10-Hydroxyoctadecanoic	$CH_3-(CH_2)_7-CHOH-(CH_2)_8-COOH$	human faecal lipids	115
$C_{18}H_{34}O_3$	cis-9,10-Epoxyoctadecanoic	$CH_3-(CH_2)_7-\begin{array}{c} \diagup \diagdown \\ \diagdown \diagup \end{array} CH-CH-(CH_2)_8-COOH$	<i>Tragopogon porrifolius</i>	119
$C_{18}H_{32}O_3$	Coronaric	$CH_3-(CH_2)_8-CH=CH-CH_2-CH-CH-(CH_2)_7-COOH$ 	<i>Puccinia graminis</i>	67
$C_{18}H_{32}O_3$	Vernolic	$CH_3-(CH_2)_8-CH=CH-CH_2-CH-CH-(CH_2)_7-COOH$ 	<i>Chrysanthemum coronarium</i>	120
			<i>Vernonia anthelmintica</i>	121, 122
$C_{18}H_{32}O_3$	Dimorpholic	$CH_3-(CH_2)_8-CH=CH-CH=CH-CHOH-(CH_2)_7-COOH$	other species	120, 123, 124, 125
$C_{18}H_{32}O_3$	cis-15,16-Epoxylinoleic	$CH_3-CH_2-CH-CH-(CH_2)_7-CH=CH-(CH_2)_7-COOH$ 	<i>Dimorpholthea aurantiaca</i>	66
			<i>Camelina sativa</i>	126
$C_{18}H_{36}O_4$	9,10,18-Trihydroxyoctadecanoic	$HOCH_2-(CH_2)_7-CHOH-CHOH-(CH_2)_8-COOH$	olive leaf oil	112
$C_{20}H_{38}O_3$	Eicosa-8,11,14,17-tetraenoic	$CH_3-(CH_2-CH=CH)_4-(CH_2)_8-COOH$	menhaden body oil	95
$C_{20}H_{38}O_3$	Eicosa-5,8,11,14-pentaenoic	$CH_3-(CH_2-CH=CH)_4-(CH_2)_7-COOH$	beef heart lipids	127
$C_{22}H_{40}O_3$	Docosa-7,10,13,16,19-pentaenoic	$CH_3-(CH_2-CH=CH)_5-(CH_2)_6-COOH$	menhaden body oil	95
$C_{28}H_{50}O_3$	Aparajitine	$CH_3-(CH_2)_{19}-CH-CH_2-CH(CH_3)-CH_2-CO$ 	herring oil	128
			<i>Clitoria ternatea</i> , <i>Clitoria maritima</i>	128a
$C_{30}H_{50}O_3$	Nocardic	$CH_3-(CH_2)_7-CH=CH-(R)-CH(OH)-CH(CH_3)-COOH$	<i>Nocardia asteroides</i>	129

* c and t designates the stereochemistry (cis or trans) of the double bond.

parinaric acids (which contain four conjugated double bonds) has been investigated (138).

The 2,4-decadienals, obtained from palm oil or ground nut oil, are a mixture (28:72) of the *trans,trans* and *trans-2,cis-4* isomers; these aldehydes probably arise from auto-oxidation of linolenic acid (139).

Fatty acid derivatives.—The fatty acid derivative 2,2-dimethoxypropane has been used in the preparation of methyl esters (140). Epoxy acids (141, 142) and poly- α -dihydroxy acids (143, 144) have been prepared by conventional procedures from various unsaturated acids. *Cis*-epoxy acids are reduced by LiAlH_4 in ether into diols, whereas, under the same conditions, *trans*-epoxy acids give epoxy alcohols (144a). Derivatives of 9,12-linoleic acid have been obtained through their bromine adducts (145). A simplified procedure for preparing 9,12-dioxo-*trans*-10-octadecenoic acid from ricinoleic acid has been devised (146). The 8-*trans*,10-*trans*-octadecadienoic acid has been isolated from the mixture of unsaturated acids arising from the action of N-bromosuccinimide on oleic acid (147). 10-Hydroxydecanoic acid can be conveniently prepared by ozonolysis of undecylenic acid (148). Chaulmoogra aldehyde has been prepared from the corresponding acid through an intermediate diazoketone (149).

Structural determination of fatty acids.—Two methods have been devised for determining the location of a branch in a saturated carbon chain of fatty acids: oxidation either by chromic acid (150) or by potassium permanganate (151). In both cases, oxidation products (methylketones and mono- and diacids) are analyzed by vapor-phase chromatography.

Ozonolysis is not an entirely satisfactory procedure for determining the position of olefinic linkages because ozonides undergo secondary reactions (152). The effect of solvent on the ozonisation of methyl oleate has been studied; methanol or acetic acid are among the best reaction media (153).

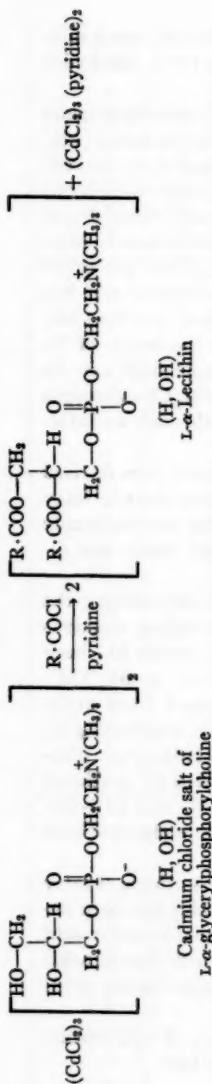
Synthesis.—Long-chain fatty acids have been synthesized from undecylenic acid through an acetylenic intermediate (154). Chain-lengthening by Kolbe electrolytic synthesis has been reviewed by Weedon (155). New illustrations of the fecundity of acetylenic chemistry are provided by syntheses of linoleic, γ -linolenic, docosa-4,7,10,13,16-pentaenoic (156), and (\pm)-vernolic acids (157). Several keto acids, possible intermediates in the synthesis of various fatty acids, have been prepared (158 to 161).

A simplified preparation of a zinc-copper couple for the synthesis of fatty acids that contain a cyclopropane ring (e.g., lactobacillic acid) has been devised (162) and used in the preparation of sterculic acid from stearolic acid (165). Syntheses of DL-10-methyloctadecanoic acid (tuberculo-stearic acid) (163) and *erythro*-2,4,6-trimethyl-2-tetracosanoic acid (mycolipenic acid) (83, 164) have been described.

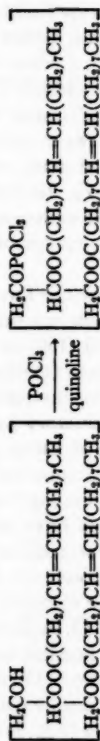
The complete series of normal higher primary alcohols of odd carbon number from undecanol to pentacosanol has been prepared (166).

PHOSPHOLIPIDS

Syntheses.—Saturated and unsaturated L- α -lecithins can be obtained from L- α -glycerylphosphorylcholine by acylation of its cadmium chloride

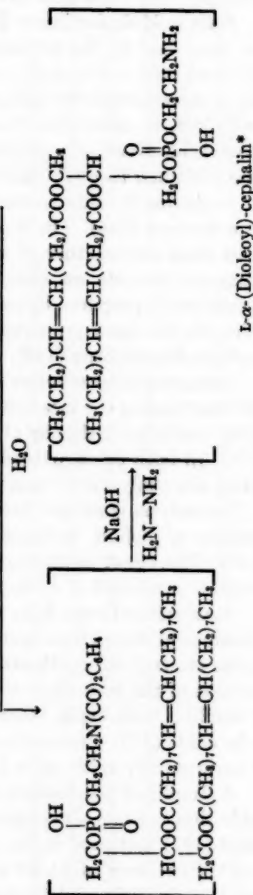


Reaction 1*



D-α,β-Diolenin

2'-hydroxyethylphthalimide + pyridine



Reaction 2

* D- And DL-α-(diolenyl)-cephalin can be obtained by the same procedure by using as starting materials L- and DL-α,β-diolenin, respectively.

addition compound with fatty acid chloride in pyridine (167) (Reaction 1).

L- α -(Dioleoyl)-cephalin has been obtained by phosphorylation of D- α , β -diolein with phosphorous oxychloride in quinoline, immediate esterification of the resulting L- α -dioleylglycerylphosphoryl dichloride with 2'-hydroxyethylphthalimid in pyridine, and removal of the protective phthaloyl group by hydrazinolysis (168) (Reaction 2).

Baer *et al.* (169) have obtained L- α -glycerylphosphoryl-L-serine by esterification of L- α -glycerylphenylphosphorylchloride with N-carbobenzoxy-L-serine benzyl ester and removal of the protective group by catalytic hydrolysis and acid hydrolysis. The optical purity of all these compounds was checked. Baer & Mahadevan (170) have synthesized water-soluble glycerol-phosphatides, i.e., didecanoyl-, dioctanoyl-, and dihexanoyl-L- α -lecithins.

The synthesis of a "mixed acid- α -lecithin" i.e., γ -octadecanoyl- β -dodecanoylglycerol- α -phosphorylcholine, starting from γ -octadecanoyl- β -dodecanoyl- α -iodohydrin has been described by de Haas & Van Deenen (171).

Hoefnagel *et al.* (172) have described a new synthesis of glycerol- α -cephalins and glycolcephalins. The corresponding silver benzyldiacylglycerol- α -phosphate or silver benzylglycolphosphate is boiled in benzene solution with 2-(dibenzylamino)-ethylbromide, and the O,N,N-tribenzyl derivative of the cephalin thus obtained is converted into the cephalin by hydrogenolysis.

Bevan & Malkin (173) have synthesized the β -batyl-(glycerol-2-octadecyl ether) analogue of cephalin, and melting-point data for batyl- and its derivatives are given.

The synthetic work of Shapiro and his colleagues, part of which was reviewed last year (174), has culminated in the synthesis of dihydrosphingomyelin (175) and, finally, of sphingomyelin (176).

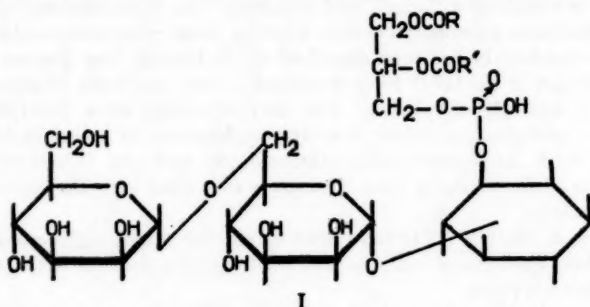
Natural phospholipids.—Hübscher & Clark (177) have given clear evidence for the natural existence of phosphatidic acids in mammalian liver and have described an isolation procedure for such compounds. Six to 7 per cent of these seem to be present as plasmalogen; between 80 and 90 per cent of the fatty acids of these phosphatidic acids are unsaturated, the majority being linoleic acid. Hokin & Hokin (178) have reported the enzymatic synthesis of a lysophosphatidic acid from monoglyceride and ATP.

Tattrie (179) has used an ingenious method to determine the position of saturated and unsaturated fatty acids in egg lecithin: after enzymatic hydrolysis with lecithinase D to the corresponding diglyceride, myristic acid was esterified to the free hydroxyl group, and the resulting triglyceride was hydrolysed with pancreatic lipase, which specifically cleaves fatty acids from the α - and α prime-positions. Myristic, palmitic, and stearic acids were freed by this enzymatic hydrolysis, proving that in the egg lecithin the saturated acids are on the α prime-position, while the unsaturated acids are in the β -position. Bremer & Greenberg (180) have found that mono- and dimethylethanolamine are constituents of rat liver phospholipids.

Gjone *et al.* (53) have isolated lysolecithin by silicic acid chromatography of lipids of normal human serum. Svennerholm & Thorin (181) have reported

on the isolation of "cephalin B" from cerebral lipids; this compound is very similar to the phospholipid from egg yolk studied by Carter *et al.* (75), which is a glycerol ether phospholipid consisting mainly of a phosphoryl ethanolamine derivative of batyl alcohol.

The position of the fatty aldehyde in an ethanolamine plasmalogen of brain has been studied by Debuch (182). Pure batyl and chimyl alcohols could be isolated after hydrogenation and hydrolysis; this shows that, in the original phospholipids, the aldehydes are bound to the alpha-position of glycerol. From an analysis of the plasmalogen of normal and neoplastic tissues of man, rabbit, and rat, Rapport & Lerner (183) conclude that almost all plasmalogens of mammalian organs have, as a common structural feature, an alpha-unsaturated ether linkage in a relatively long fatty chain. A lyso-



phosphatidylcholine has been obtained by Hartree & Mann (184) from an alkaline hydrolysate of the lecithin fraction from ox heart; the ratio phosphorus:choline:fatty aldehyde in this preparation is 1:1:1.

Lewin & Wagenknecht (185) have studied isolation and purification methods for an inositol phosphatide fraction of peas. Brown *et al.* (186) have elaborated methods for establishing the position of linkage of inositol residues in the natural phosphoinositides. Glycerolinositol phosphate can be oxidized by periodate, and then treated by phenylhydrazine to yield the corresponding inositol phosphates without significant phosphate migration. The structure of a phosphatide isolated from crude corn has been established as 1-phosphatidyl-*myo*-inositol (186a). A methyl ether of *d*-inositol (pinitol) has been characterized in the hydrolyzate of soy bean phosphatides, which are difficult to extract (186b).

The principal phospholipid of mycobacteria is a phosphatidylinositol-*D*-mannobioside; a 6-*O*- α -*D*-mannopyranosido- α -*D*-mannopyranose is linked glycosidically to one of the hydroxyl groups of inositol. Vilkas & Lederer (187) have proposed as a possible structure Formula I, in which the point of attachment of the mannobiose remains undetermined. A phosphatidylinositolpentamannoside of mycobacteria has been described by Nojima (188) and Pangborn (189), and a phosphatidylinositolpentaglucoside, by Vilkas (190).

Cardiolipin.—Macfarlane & Gray (191, 192) have shown that cardiolipin

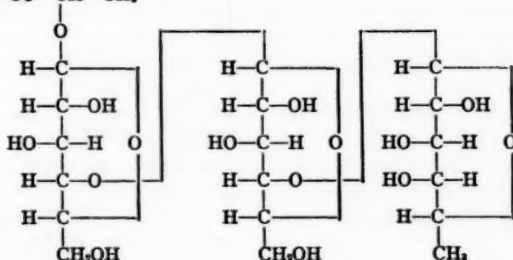
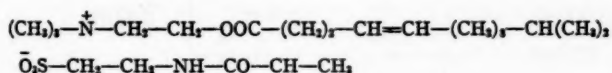
obtained from ox heart is composed of two phosphoric acid, three glycerol, and four fatty acid residues. The polyglycerolphosphate skeleton $[\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{O}-\text{P}=\text{O}(\text{OH})-\text{OCH}_2-\text{CHOH}-\text{CH}_2\text{O}-\text{P}=\text{O}(\text{OH})-\text{OCH}_2-\text{CHOH}-\text{CH}_2\text{OH}]$ thus formed has five hydroxyl groups available for esterification. Macfarlane & Wheeldon (193) have shown that the terminal glycerol residues are esterified as diglycerides, leaving a free hydroxyl group on the middle glycerol residue. A cardiolipin-like compound seems to exist also in rat liver mitochondria (194). Benson & Strickland (195) have found a quite similar phospholipid in *Chlorella*, *Rhodospirillum rubrum*, and higher plants; on deacylation it gives a 1,3-diglycerophosphorylglycerol.

Sphingomyelin.—Synthetic work on sphingomyelin has been mentioned in the foregoing paragraphs. Fujino & Negishi (196) have reported the presence of behenic acid in sphingomyelin from horse spinal cord. Proštenik & Majhofer-Orešćanin (196a) have obtained analytical evidence for the existence of a C_{20} sphingosine in the sphingolipid bases from horse and beef brain.

GLYCOLIPIDS

Law (174) last year reviewed the chemistry of glycolipids; a detailed review of glycolipids of acid-fast bacteria has been published recently (197).

A very particular glycolipid, which was first obtained by Akiya & Nakazawa (198) from oysters, has been analyzed by Nakazawa (199); this compound, $\text{C}_{44}\text{H}_{82}\text{N}_2\text{O}_{21}$, on hydrolysis gives equimolecular quantities of choline, 14-methylpentadec-4-enoic acid, lactic acid, taurine, and a trisaccharide that was identified as D-glucopyranosyl- α -D-glucopyranosyl-L-fucopyranose. The structure shown in Formula II was finally proposed for this glycolipid, which can be split by strong ionic exchangers into two components: one contains choline and the fatty acid; the other, N-lactyltaurine and the trisaccharide.



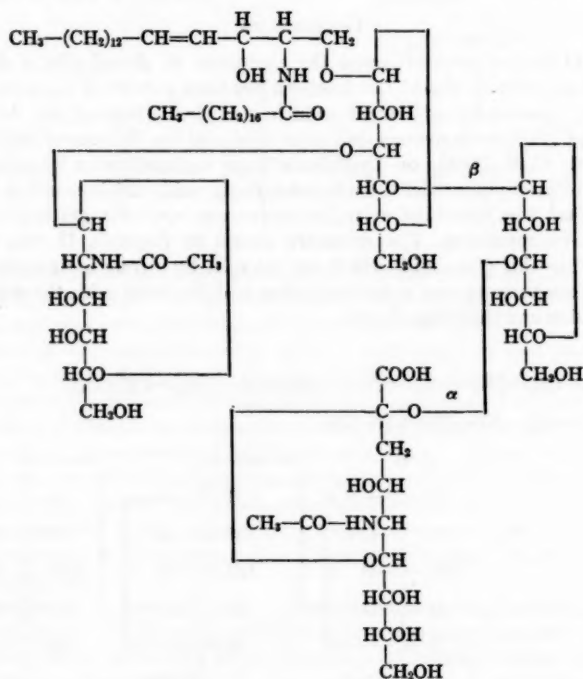
II

Cerebrosides.—Marinetti *et al.* (200) have isolated crystalline cerebrosides from the spleen of a woman with Gaucher's disease. The sugar moiety was

glucose and its position on the primary hydroxyl group of sphingosine was established; the fatty acids of the cerebrosides were mainly lignoceric, behenic, and palmitic acids.

Gangliosides.—Detailed formulae for gangliosides have been recently developed by two groups of workers. Klenk & Gielen (201) have described a ganglioside of human brain that contains hexosamine. The primary hydroxyl of N-octadecanoyl sphingosine is glycosidized by a tetrasaccharide that has the structure of an N-acetyl-3-neuraminyl-N-acetyl-3-galactosaminyl-4-galactosyl glucose. A second ganglioside containing no hexosamine but one molecule of N-acetyl neuraminic acid was also isolated.

Kuhn *et al.* (202) have isolated pure crystalline gangliosides from ox brain. One of these, G_2 , gave on hydrolysis equimolecular amounts of stearic acid, sphingosine, glucose, galactose, N-acetyl galactosamine and N-acetyl neuraminic acid; the results of permethylation studies and of acetolysis are in agreement with Formula III.



III

Cytolipin H.—Rapport *et al.* (203) have investigated the structure of cytolipin H, a lipid hapten that accounts for most of the antilipid reactivity of antisera prepared in rabbits by injection of human tumor fractions. The

material was isolated from human epidermoid carcinoma; it is a glycosphingolipid that contains fatty acids, sphingosine, glucose, and galactose.

Mycosides.—Mycobacteria contain several glycolipids that seem to be specific for certain types of these bacteria (204); they are characterized by the presence of O-methylated 6-deoxyhexoses in glycosidic linkage (205). The proposal has been made to call these compounds "mycosides" and to define them as "type specific glycolipids of mycobacterial origin" (206). Three mycosides have been described to date: mycoside A (formerly called compound G_A), which was present in all 17 photochromogenic strains studied; mycoside B (formerly called compound G_B), which was present in all 7 bovine strains studied; and mycoside C (formerly called compound J_{AV}), which was present in 11 out of 13 avian strains studied (204).

Mycoside A contains three different O-methylated 6-deoxyhexoses, which have been identified as 2-O-methyl fucose, 2-O-methyl rhamnose, and 2,4-di-O-methyl rhamnose (205). The lipid moiety is a di- or trimycocerosate of an aromatic alcohol with a typical ultraviolet spectrum.

Mycoside B has about the same ultraviolet spectrum as mycoside A; the probable molecular formula is C₃₂H₁₃₂O₁₀. It contains only one sugar, identified by MacLennan *et al.* (205) as 2-O-methyl rhamnose. The lipid moiety has the formula C₇₅H₁₄₀O₈ and is a diester of two molecules of a branched-chain acid fraction of mean molecular weight 340 (C₂₂H₄₄O₂) with a methoxylated phenolic triol, C₃₁H₅₆O₄. The deoxyhexose is linked glycosidically to the phenolic hydroxyl group of the lipid moiety (207).

Mycoside C is a peptidoglycolipid and will be mentioned later.

AMINO ACID-CONTAINING LIPIDS

Lipoamino acids.—In recent years, several workers have studied the formation of liposoluble amino acid derivatives in biological systems. It has even been surmised that amino acids may pass through a lipid-bound stage before incorporation into proteins (208). In some instances, it was observed that the amino acid is held by a highly labile (electrostatic?) bond in the lipid fraction (208), whereas Haining *et al.* (209) have found that prolonged acid hydrolysis of the non-phosphatide lipid (of rat liver microsomes) is necessary to liberate the incorporated ¹⁴C-phenylalanine. This property suggests an amide linkage of a fatty acid with the amino group of the phenylalanine.

More recently, Fukui & Axelrod (210) have reported the formation of N-oleoylphenylalanine by a rat liver enzyme. Gaby & Silbermann (211) have also studied the incorporation of labeled amino acids into the phospholipids of rat liver slices; the nature of the chemical bond was not defined. Wren (212) has studied the experimental conditions under which the amino acids that are added *in vitro* are bound to phospholipids.

Peptidolipids.—Peptidolipids are liposoluble compounds that contain a peptide moiety and a lipid moiety in covalent linkage. They should be distinguished from water-soluble lipopeptides [such as the antibiotic circulin, which is an oligopeptide that contains a relatively small lipid moiety, *e.g.*, 6-methyl octanoic acid (213)].

Guinand *et al.* (214) have described a peptidolipid fraction isolated from the actinomycete *Nocardia asteroides*; it is very soluble in chloroform, insoluble in ether and in water, and contains 30 per cent of a lipid moiety (a mixture of C_{22} saturated hydroxy acids) that is linked by an amide linkage to an oligopeptide containing the following six amino acids: threonine, alanine, valine, isoleucine, leucine, and proline.

PEPTIDOGLYCOLIPIDS

Ikawa *et al.* (215) have drawn attention to the fact that bacterial peptidolipids and peptidoglycolipids frequently contain D-amino acids, which until recently have been found only in antibiotics and microbial cell walls.

Mycoside C differs from the other mycosides mentioned above by its nitrogen content, which is due to the presence of several amino acids. Mycoside C is a mixture of closely related peptidoglycolipids which can be separated by chromatography on silicic acid (216). They contain three different amino acids linked in a pentapeptide: one molecule of D-phenylalanine, two molecules of D-*allo*-threonine, and two molecules of D-alanine; D-*allo*-threonine had not previously been isolated from natural sources. The pentapeptide has the structure: D-Phe·D-*allo*-Thr·D-Ala·D-*allo*-Thr·D-Ala.

All mycoside C preparations contain three different deoxyhexoses: 6-deoxytalose, 3-O-methyl-6-deoxytalose, and 3,4-di-O-methyl rhamnose. The lipid moiety of these mycoside C preparations seems to be a mixture of saturated and unsaturated hydroxy acids. Two O-acetyl groups are also present in each of these fractions.

Human strains of mycobacteria contain a high-melting acetone-insoluble fraction called wax D (217), which can replace whole killed mycobacteria as immunological adjuvants in the Freund-adjuvant (218). The wax D of the strain *Brévannes* has a molecular weight of about 54,000 and contains a heptapeptide linked to a carbohydrate moiety (mol. wt. about 26,000) which is esterified by approximately 22 molecules of mycolic acid ($C_{88}H_{176}O_4$) (219).

The structure of the heptapeptide has been determined as: *meso*-DAP*·D-Ala·D-Glu·D-Glu·L-Ala·*meso*-DAP·L-Ala (*DAP= α,ϵ -diaminopimelic acid). Again, we note a remarkable accumulation of D-amino acids (220). It might be recalled that the cell wall of mycobacteria contains the same three amino acids: α,ϵ -diaminopimelic acid, Glu, and Ala (221). One of the *meso*- α,ϵ -diaminopimelic acid molecules is linked through one of the carboxyl groups to galactosamine, which itself is linked glycosidically to D-arabinose. Thus, galactosamine is the bridge between the peptide and the carbohydrate moiety. A second linkage to the carbohydrate is formed by an ester between one of the carboxyls of D-Glu and arabinose (220).

LIPIDS OF MICROORGANISMS

Moulds and yeasts.—Fat production by microorganisms has been reviewed by Woodbine (222), and the composition of the lipids of yeasts has been reported by Eddy (223). Unusual features have been found in the extracellular lipids produced by *Rhodotorula graminis* (up to 3 to 4 gm/l culture medium):

sorbitol and arabitol are the only hydrosoluble components; intracellular lipids contain sorbitol and glycerol (224).

Bacteria.—The composition of bacterial lipids has been reviewed by Asselineau & Lederer (225); reviews limited to the lipids of mycobacteria have been published by Pokorny (226) and Lederer (227). Infrared spectroscopy has been extensively used by Smith, Randall, and others (204) to detect the presence of type specific compounds in various species of mycobacteria; this study led to the discovery of the mycosides (see above.) Vapor-phase chromatography has been used to study the composition of the fatty acid mixture isolated from the lipids of *Mycobacterium tuberculosis* (22, 35, 228), *Escherichia coli* (113), *Corynebacterium diphtheriae*, *Corynebacterium ovis*, *Pasteurella pestis*, and *Bacillus subtilis* (114); composition has been found to vary by genus.

Mycobacteria.—Investigations of the structure of mycocerosic and mycolic acids, phosphatides, mycosides, and wax D have been mentioned above. Sixty per cent of lipids have been found in the cell wall of BCG (229). Several N-mycoloyl amino acids have been prepared; they are non-toxic for mice but may cause a significant decrease in succinic dehydrogenase activity in liver homogenates (230).

Corynebacteria.—The cell wall of *Corynebacterium diphtheriae* has been studied extensively (231, 232, 233); toxic properties are exhibited by some fractions (233). Corynomycolic acid has been found in the lipids of *Corynebacterium ovis* (234); it might be a typical component of the lipids of corynebacteria species (as are mycolic acids in mycobacteria).

Brucella.—The lipids of three species of brucella have been isolated (235, 236); glycolipids are present (236).

Escherichia coli.—The presence of a C₁₇ fatty acid that contains a cyclopropane ring has been demonstrated (113), and phospholipids that contain ethanolamine and serine, but no choline, have been isolated from *E. coli* (237).

Miscellaneous species.—Poly- β -hydroxybutyrate is present in rhizobium and spirillum species (238), *Rhospirillum rubrum* (239), and a few species of pseudomonas (238, 240, 241). The lipids of *Bacillus stearothermophilus* (242) and of the L form of *Proteus morgani* (243) have been investigated. Reports on the absence of sterols in bacteria have been published on micromonospora species (244), *Rhodospirillum rubrum* and *Streptomyces griseus* (244), three species of brucella (235), and *Bacillus stearothermophilus* (242).

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X-RAY STUDIES OF COMPOUNDS OF BIOLOGICAL INTEREST^{1,2}

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It has been four years since the previous review of this subject [Kendrew & Perutz (1) appeared in the *Annual Review of Biochemistry*. In some ways, the present review marks a turning point in our knowledge of the molecular structure of biological systems. Globular proteins are probably the most complicated molecules found in biology, and here we will cover the work that has led to our first three-dimensional understanding of the structure of myoglobin and hemoglobin. It is hard to overestimate the importance of this knowledge to a fundamental understanding of biochemistry. As molecules get larger and more complex, it becomes increasingly difficult to understand chemical behavior without a clear comprehension of exact geometric relations. We are now approaching this kind of geometric understanding because of the crystallographic protein structure work. This work is important because a complete knowledge of molecular geometry will ultimately make it possible to understand a large variety of molecular properties, such as enzymatic activity, antigenicity, etc.

This review is divided roughly into three parts. In the first part we discuss the work that has been carried out on amino acids, peptides, and fibrous and globular proteins. Our main interest will be focussed on the globular proteins; limitations of space prevent our treating in detail several crystal structure determinations of relevant small molecules. In this section, we shall try to draw the attention of the biochemist to the fact that the solution of additional globular protein structures is no longer impeded by crystallographic difficulties but rather by chemical limitations.

The next section deals with studies of crystalline nucleic acid derivatives, nucleic acids, synthetic polynucleotides, nucleoproteins, and finally viruses. The final and smallest section of the review briefly covers sugars, polysaccharides, and other items.

X-RAY DIFFRACTION AND BIOLOGICAL MATERIALS

An excellent review of the application of x-ray analysis to biological problems has been published by Crick & Kendrew (2). Most of the problems

¹ The survey of the literature pertaining to this review was concluded in October, 1960.

² The following abbreviations are used: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; TMV for tobacco mosaic virus.

faced by investigators in biological crystallography are shared by other crystallographers. The fundamental problem arises from the fact that x-rays, like any other wave motion, are characterized by an amplitude and a phase, both of which must be known in order to deduce the molecular structure of the material that is diffracting the x-rays. X-rays register on film in the form of a blackening which is related to the amplitude; however, phase information is lost and must be supplied by various stratagems. Interatomic vectors can be calculated directly from the intensity in the form of the Patterson function, but this does not lead to the molecular structure, except in the case of some of the simplest of amino acids or sugars. More complicated molecules require other techniques. Heavy atoms that contain a large number of electrons, such as iodine or mercury, can be used in an attempt to obtain phase information. The work of Hodgkin *et al.* (3) on vitamin B₁₂ is a good example of the use of the heavy atom method, since the cobalt atom located at the center of the porphyrin ring could be used directly. The position of the cobalt atom was fixed from the Patterson function. Then, by using the phases of the cobalt atom together with the amplitudes from the entire molecule, it was possible to calculate a complete electron density distribution. From this initial calculation, parts of the porphyrin ring could be seen, and these additional new parts could be used for determining phases in the next calculation. In this way, a successive series of calculations gradually revealed the molecule in its entirety.

While this method is useful for small or medium-sized molecules, it is ineffective for really large structures, such as proteins or viruses. In these cases, heavy atoms can also be used in the isomorphous replacement method. This method was discussed somewhat in the previous review by Kendrew & Perutz (1) and will be described below in the section on globular proteins.

Another part of biological crystallography involves the study of materials that often are non-crystalline. These include fibers such as fibrous proteins, nucleic acids, or polysaccharides. Here a substantial difficulty is that of obtaining high orientation along the fiber axis. These fibers are usually polymers and the solution of their molecular structure is often accomplished by intuition suitably buttressed with aids, such as molecular models.

In considering fibrous structures, there are two considerations of importance. Since the materials are polymers, there is considerable restraint on the position in space of the molecular units, as they must connect with each other to form a molecular chain. This substantially reduces the number of potential molecular models under consideration. Furthermore, these chains are often helical when they have a periodic configuration. The theory of helical diffraction has been worked out in considerable detail (4, 5), and it has been widely applied. It is also important to realize that with fibers there is usually insufficient experimental x-ray data to determine unambiguously the position in space of all atoms. However, the molecular geometry of the individual polymer components, amino acids, sugars, or nucleotides is usually taken from the results of single crystal analysis. Thus, the x-ray diffraction data

from fibers are not usually used to deduce the bond angles and interatomic distances of the monomer units; these are assumed, and the fiber diffraction data are used to establish the position in space of the individual units relative to each other.

AMINO ACIDS AND SMALL PEPTIDES

Table I lists the crystal structure determinations which have been carried out on the twenty amino acids commonly found in proteins. Where more than one analysis has been made for any particular crystal form, only the most accurate result has been quoted. An example is α -glycine, the crystal structure of which was first determined more than twenty years ago, with a

TABLE I
CRYSTAL STRUCTURE ANALYSIS OF AMINO ACIDS AND PEPTIDES

Compound studied	Crystallographic data*	Maximum error†	References	Peptides analyzed by x-rays‡
α -Glycine	3D	0.003 Å	6	α -Gly-Gly; β -Gly-Gly;
β -Glycine	Two 2D	0.05 Å	7	Glutathione; Gly-L-Ala;
γ -Glycine	3D		8	Gly-L-Try; Cys-Gly-NaI;
				Gly-L-Tyr; L-Leu-L-Pro-Gly;
				N,N'-diglycyl-L-cystine·2H ₂ O
DL-Alanine	3D	0.02 Å	9	Gly-L-Ala
DL-Valine	Cell dimensions and space group only		10	—
DL-Leucine	Cell dimensions and space group only		10	L-Leu-L-Pro-Gly
D-Ileu·HCl·H ₂ O	Two 2D	0.12 Å	11	—
D-Ileu·HBr·H ₂ O				—
DL-Serine	3D	0.02 Å	12	—
L-Serine phosphate	3D	0.02 Å	13	—
L-Threonine	3D	0.02 Å	14	—
Phenylalanine	Cell dimensions and space group only		15	—
L-Tyr·HBr	Two 2D	0.10 Å	16	Gly-L-Tyr·HCl
L- β -Glutamic acid	Three 2D	0.05 Å	17	—
L-Glutamine	Two 2D	0.05 Å	18	—
L- $\begin{pmatrix} \text{Zn} \\ \text{Co} \\ \text{Ni} \end{pmatrix}$ ·Asp·3H ₂ O	3D		19	Gly-L-Asp
L-Lys·HCl·2H ₂ O	Two 2D	0.15 Å	20	—
L-Copper proline·2H ₂ O	Three 2D	0.12 Å	21	L-Leu-L-Pro-Gly
L-Hydroxyproline	3D	0.03 Å	22	—
His·HCl·H ₂ O	3D	0.03 Å	23	—
Tryptophan	No data	—	—	Gly-L-Try·2H ₂ O
DL- α Methionine	Two 2D	0.05 Å	24	—
DL- β Methionine				
L-Cystine	3D	0.007 Å	25	N,N'-diglycyl-L-cystine·2H ₂ O
L-Cystine·2HBr	Two 2D	0.06 Å	26	—

* In specifying the crystallographic data, 3D denotes the use of a complete three-dimensional set of intensity measurements; Two 2D, the use of two two-dimensional sets.

† The error quoted is a reasonable maximum, that is, three times the standard deviation assuming a random distribution.

‡ References for the peptide analyses are given in the text and in Kendrew & Perutz (1).

standard error of 0.04 Å in the atomic co-ordinates (27). This structure has now been greatly refined by using extended data and modern computing methods; the standard error has thus been reduced to 0.001 Å (6). A similar list was prepared four years ago by Kendrew & Perutz (1); data have since become available for three more of these amino acids: lysine, aspartic acid, and tyrosine. Also, structure determinations have been reported for hexagonal L-cystine and cystine dihydrobromide, for two further polymorphs of glycine, and for serine phosphate. The crystal structures of two tripeptides, glutathione (γ -glutamyl-cysteinyl-glycine) and L-leucyl-L-prolyl-glycine, have also been reported in detail since the last review.

Several of the determinations have been made with three-dimensional data, which give sufficient accuracy in the determination of atomic co-ordinates for slight variations of bond lengths to be truly significant. Hahn (28), from this point of view, has made a careful comparison of the structures available in 1957. He found that the C—NH₃⁺ bond in amino acid and peptide zwitterions is always some 0.028 Å longer than the standard C—N bond of 1.475 Å and has proposed some explanations of this lengthening. His review of peptide bond angles and distances has enabled him to deduce a consistent set within the regular polypeptide chain. It is reassuring to see that these differ only slightly from the dimensions which were originally given by Corey & Pauling (29) and which have been widely used as canonical dimensions of the α -helix and other polypeptide chain configurations. Another axiom of polypeptide structure is the planarity of the peptide (and amide) group associated with a C—N bond length of 1.32 Å. This is accurately verified in the crystal structure analyses of N-methyl acetamide (30) and diketopiperazine (31). In one crystal form of N-methyl acetamide, the amide group atoms have been shown to be coplanar to within 0.015 Å, whereas in diketopiperazine the six atoms of the ring and the two C α -atoms are coplanar to within 0.010 Å. The bond angles in diketopiperazine are somewhat strained from their usual values, and there is evidence of a conjugation effect in the shortening of the bond lengths within the ring. However, in N-methyl acetamide, all the dimensions are very close to those adopted as standard by Corey & Donohue (32), except the C—N peptide bond which appears shortened by 0.03 Å.

The planarity of the peptide groups, with C=O and N—H bonds in the *trans* configuration, is the most striking feature common to the two tripeptides, L-leucyl-L-prolyl-glycine (33) and glutathione (γ -L-glutamyl-L-cysteinyl-glycine) (34). In any such structure, the configuration of the peptide chain may conveniently be described in terms of the twists about the C α —N and C α —C bonds within one amino acid away from the *cis* arrangement of N—H and C=O bonds. In leucyl-prolyl-glycine all the twists are small except that about C α —N of the pyrrolidine ring, which is fixed by the geometry of the ring at 102°. All the atoms of the peptide chain lie close to two planes which intersect in this C α —N bond. The *cis* arrangement of C=O and N—H bonds across C α has been observed in several amino acid and

dipeptide structures and is an important feature of the β -type extended polypeptide chain. Thus, finding this configuration in leucyl-prolyl-glycine probably means that it is self-determined rather than imposed by any requirements of the crystal structure, even though there are no intramolecular hydrogen bonds to stabilize it.

In addition to information on the configuration of the backbone atoms in polypeptide chains, crystal structure studies of comparatively small peptides may provide examples of side-chain interactions which stabilize protein molecules. Evidence on the configuration of the disulphide bridge of cystine is already available from three different crystal structure determinations: N,N'-diglycyl-L-cystine (35), hexagonal L-cystine (25), and L-cystine dihydrobromide (26). In these, the molecule is symmetrical with a twofold axis passing through the center of the S—S bond. Also, the bond lengths and angles are much the same in the three structures: S—S = 2.03 Å, the angle at the S-atom 105°, and the C—S—S—C dihedral angle is 100°. The configurations in N,N'-diglycyl L-cystine and L-cystine dihydrobromide are altogether similar, but in hexagonal L-cystine the C—S—S—C bridge has the mirror-image configuration.

For the various side-chain interactions through hydrogen bonding, such as those postulated by Scheraga in his model of ribonuclease (36), there do not appear to be any directly relevant crystal structure models. Studies of suitable peptides would certainly be valuable, for in several cases there appear to be a limited number of possible configurations. For the interactions between proteins and metal ions, crystal structure studies can suggest possible configurations around the metal atom. Among the recently determined crystal structures that are of interest in this way are two complexes of copper and biuret (37) and a complex of biuret and cadmium chloride (38).

POLYPEPTIDES AND FIBROUS PROTEINS

There is a close relation between the molecular structure of synthetic polypeptides and the naturally occurring fibrous proteins. This has been seen most clearly in x-ray diffraction studies. This aspect of the subject was covered in considerable detail in the last review by Kendrew & Perutz (1); accordingly, we will only give a brief discussion here. A short review of this subject has been published recently by Rich (39).

Synthetic polypeptides.—The most widely known configuration of polypeptide chains is the α -helix, first proposed by Pauling, Corey & Branson in 1951 (40). Although there had been a general correspondence between the x-ray diffraction pattern predicted by this configuration and that observed by a variety of synthetic polypeptides, a detailed and satisfactory agreement had not been reported at the time of the last review. Poly γ -L-methyl glutamate was first used as an example of the α -helix, but a detailed examination of the diffraction pattern revealed several discrepancies, most of which arose from the fact that the side chain of the helix is very large (41). To overcome this difficulty, work has been concentrated on the α form of poly-L-alanine.

Brown & Trotter (42) have carried out a detailed comparison between the observed diffraction pattern of polyalanine and the predictions of the α -helix. They showed that the unit cell is hexagonal with 8.55 Å on a side and that there was quite good agreement between observed and calculated intensities for the equatorial reflections, but that, in general, the agreement was poor elsewhere on the diagram. However, Elliott & Malcolm (43) took up this study and, recognizing that the α -helix is a polar structure, sought to investigate the effects of having a random up and down arrangement of α -helical chains. This can be done because the position of the methyl group on the alanine residues is roughly the same irrespective of the orientation of the main helix. Using a model of this type, they found a substantially better agreement between the polyalanine x-ray diffraction pattern and the calculated intensities, and furthermore, showed that the best agreement was reached with right-handed α -helices. This is in accord with some of the results obtained from optical rotatory dispersion studies. At the present time, this work represents the best agreement between observed and calculated quantities for the α -helix in any synthetic polypeptide.

Further work has been done by Sasisekharan (44) on refining the structure of poly-L-proline-II. Using the configuration that had been worked out by Cowen & McGavin (45), he found how the chains were packed together in the crystal. In an additional study, he worked out the crystal structure of poly-L-hydroxyproline (46). This polymer has the same configuration as poly-L-proline in which the molecules exist in the form of a somewhat extended helix with three residues per turn. Including the structure of polyglycine II (47), this particular configuration has now been found in three different polypeptide chains.

Work on the structure of silk was covered in detail in the review of Kendrew & Perutz. However, some additional information has been published (42); in this the β or extended form of poly-L-alanine is compared with the diffraction pattern produced by some types of silk. These have very similar unit cells and apparently both have similar types of extended pleated-sheet structures.

Collagen and elastin.—For some years there has been substantial agreement among four different groups of workers (48 to 51) that the collagen molecule consists of three coiled polypeptide strands which are helically wound round each other and which accommodate a fundamental repeating sequence, $-G-R_1-R_2-$, where G = glycine, R_1 = proline or any other amino acid, and R_2 = hydroxyproline or any other amino acid. These three chains are held together by hydrogen bonds which involve the amino group on the glycine residues but have no other systematic hydrogen bonds involving residues R_2 and R_3 . There are two closely related models, collagen I and collagen II, both of which are three-stranded coiled-coils that differ only in the phasing of the three chains relative to each other. Collagen II, in which the proline and hydroxyproline rings on positions R_2 and R_3 project away from the center of the helix axis, has been generally preferred.

Several additional studies related to the collagen structure include a book published in 1958 (52) which covers recent investigations. In this book, Rich & Crick (53) describe the derivation of collagen I and II from the structure of polyglycine II (47) and discuss various restraints on the sequence of side chains in collagen. Burge, Cowan & McGavin (54) discuss the experimental agreement between the optical and x-ray diffraction data. In a later investigation, Bradbury *et al.* (55) made a comparison of the calculated and observed diffraction data for some of the collagen layer lines and have reported new infrared studies of collagen. In addition to the physical data which lend support to the collagen II structure, a great deal of work has been done to determine the sequence of various peptides obtained from collagen by tryptic hydrolysis (56) or collagenase action (57). Both of these show that glycine residues are distributed very evenly through the hydrolytic fragments and make up a third of the residues. However, these studies also point out that the pyrrolidine rings are not distributed evenly, since some regions of the molecule are rich in these rings and others have relatively few.

Huggins (58) has proposed a different collagen model, a single-stranded helical structure which permits an extension of the chain without breaking primary bonds. However, this model does not have a calculated diffraction pattern which is in agreement with what is observed experimentally (59). Andreeva and her colleagues (60) have been carrying out x-ray diffraction experiments on collagen and have been interested in the possibility that part of the molecule exists in the form of an alternating sequence, -Gly-Pro- since one of the polypeptide fragments from collagen hydrolysis (56) contains only these two amino acids. Accordingly, they have constructed models of a -Gly-Pro- polymer (61, 62, 63). However, these models have a calculated diffraction pattern which is not in agreement with what is observed experimentally (59).

It is now five years since the triple-stranded coiled-coil model for collagen was proposed; in that time no strong contradictory data have arisen to shake one's confidence in the structure. Indeed, many feel that this aspect of the problem is largely solved. However, there are still two major structural problems under active investigation. One of these concerns the mode of aggregation of the three-stranded collagen molecules to form the larger units which are seen in tendons. That a regular organization is present has been shown by the observations of North, Cowan & Randall (64), who demonstrated a series of equatorial spacings which extend out to 47 Å. They proposed a unit cell of $62 \text{ Å} \times 76 \text{ Å}$ with an interaxial angle of 125° . Bear (51) has suggested how this cell could be filled. However, it has also been suggested by Sasisekharan & Ramachandran (65) that these spacings arise from a cylindrical packing of collagen molecules.

Another major unsolved structural problem is the origin and nature of the periodicity within the collagen molecule which gives rise to a repeating sequence of 640 Å along the fiber axis. Several studies in this area have been carried out, including a correlation of electron microscopic and x-ray observa-

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tions (66) and further low-angle x-ray studies (67). Tomlin & Ericson (68) have advanced an explanation of the occasional "fanning" of the small angle reflections that arise from the 640 Å periodicity. They point out that a shear distortion of fibrils would produce this type of pattern. Since it is possible to dissociate the elongated collagen molecules from each other and then reaggregate them to make the various types of periodicities seen in the electron microscope (69), it is quite likely that the native 640 Å periodicity arises from the amino acid sequence present in the molecule. Accordingly, it may be necessary to wait until the complete amino acid sequence of collagen has been determined before the variations in electron density along the fiber axis will be fully understood.

X-ray diffraction studies have been carried out to determine the effects of water (70) and calcium phosphate (71) on the x-ray diffraction pattern of collagen fibres. These calcification studies will undoubtedly be useful in trying to understand the mechanism of bone formation. Collagen fibrils have been found to influence the crystallization of a large number of inorganic salts (72 to 75). X-ray diffraction studies have shown that the crystallites are often deposited in an oriented array parallel to the collagen molecule. Recent work has been done (76, 77) on the reappearance of native collagen after thermal denaturation. This work suggests that the three strands in the collagen helix which separate during the formation of gelatin can be made to reaggregate under certain conditions to form the intact molecule.

Elastin is a fibrous protein closely related to collagen. Additional evidence has been presented by Ramachandran & Santanam (78) which supports Astbury's earlier hypothesis (79) that elastin is simply a denatured form of collagen. By treating collagen with some inorganic salt solutions, the material is denatured to give an amorphous x-ray powder pattern which is similar to that seen in elastin.

Keratin.—Even though there seems to be general agreement that the α -helix structure (40) is the building block in α -keratin, there is still considerable discussion concerning the packing of the α -helices to produce the keratin structures which are found in hair, horn, fingernails, and porcupine quills. Crick (80, 81) pointed out that α -helices pack most easily if their axes are tilted about 20° to each other; in this way, the side chains of adjacent helices pack next to each other so that the helices have a natural tendency to form coiled-coil structures. Pauling & Corey (82) at the same time used this concept to generate a model for the structure of α -keratin in which groups of α -helices were wrapped around each other to form seven-stranded cables or three-stranded cables packed together in a hexagonal array. An alternative model has been suggested by Huggins (83), who feels that the coiled-coil configuration would make the α - β transformation very difficult. Accordingly, he suggests a model in which polypeptide chains, probably in the form of α -helices, are arranged in groups of three and fill a large hexagonal unit cell 95 Å on an edge. Alternatively, Sasisekharan & Ramachandran (84) have sug-

gested that a cylindrical packing of small clusters of α -helical chains could explain many of the features of the equatorial diffraction pattern of α -keratin. Although there is still a great deal of uncertainty about the packing of α -helices, a significant contribution was made in electron-microscope investigations (85, 86, 87) of thin slices of the tip of porcupine quills and human hair. By use of an osmium stain, Birbeck & Mercer and Fraser, MacRae & Rogers found the entire structure to be made up of microfibrils about 70 Å in diameter which appear to pack together, sometimes in a hexagonal lattice, sometimes in a distorted hexagonal lattice, and occasionally in spiral aggregates. Thus, the structure cannot be described in simple terms. In addition, the distance between these microfibrils can be altered when the α -keratin is chemically reduced. They suggest that the α -keratin fibrils are held in a cystine-rich matrix and can be moved about when various heavy-atom staining agents are introduced into the keratin fiber.

Several studies have been carried out on the modification of the α -keratin diffraction pattern caused by the addition of various heavy metal salts (88, 89, 90). When α -keratin is either stretched or heated, the fiber elongates and produces a β -type x-ray diffraction pattern. Several studies (91 to 97) have been carried out on various details of this transformation.

The rachis of feathers is made of a keratin material which produces an x-ray diffraction pattern quite distinct from that of α -keratin (98, 99). This implies that it has a grossly different type of molecular organization which does not involve the α -helix. Krimm & Schor (100) have proposed a model for the rachis keratin, but apparently it does not predict the appropriate diffraction pattern (101). Fraser & MacRae (101) have studied the molecular organization in feather keratin by allowing the material to react with various heavy-atom stains and swelling agents; they then used a large orthogonal unit cell and generated a sheet model which is in agreement with the changes of the diffraction pattern. However, they do not propose a specific molecular configuration in this large unit cell. In summary, feather rachis keratin stands out as one of the few fibrous proteins in which the configuration of the polypeptide chains is still an unsolved problem.

Muscle proteins.—There are two x-ray problems associated with the molecular organization of muscle. One of these is related to the periodicity which gives rise to the low-angle reflections that have been reported at 420 to 430 Å, first from a marine invertebrate (102) and more recently from a fraction of light meromyosin (103). Low-angle studies have also been reported from striated muscle (104). In all cases, the origin of this periodicity is obscure. The second problem concerns the lateral packing of the α -helical chains which are believed to be a major component of muscle. It has been generally assumed that these chains are in a coiled-coil configuration since this readily explains how the near meridional 5.4 Å spacing is changed to a meridional 5.1 Å spacing (80, 81, 82). Strong experimental support for this concept has been presented by Cohen & Holmes (105) who have produced, from moist

stretched molluscan muscles, x-ray diffraction photographs which clearly suggest that the fundamental structural unit is a coiled-coil of two α -helices wound round each other with a repeat of 186 Å.

GLOBULAR PROTEINS

There has been very great progress in the x-ray analysis of globular proteins since the last review in this series (1). At that time, four years ago, some low resolution contour maps were available which showed projections onto a plane of the electron density in crystals of horse hemoglobin (106) and sperm whale myoglobin (107). The excitement which these maps produced among crystallographers could hardly be shared by most biochemists, for they showed none of the features that might have been recognized, such as the heme groups or lengths of the polypeptide chain. However, the problem was, in principle, solved at that time, since the means were at hand for extending the x-ray analysis of myoglobin to give a three-dimensional picture of the structure and with much sharper resolution. The huge task of collecting and analyzing the diffraction data was tackled with such energy by Kendrew and his collaborators that we now have a three-dimensional electron-density map for myoglobin sufficiently detailed to show not only the heme group and the polypeptide chain but even to allow a high proportion of the side chains to be identified (108). The analysis of hemoglobin was delayed for some time by the lack of suitable crystalline derivatives, but Perutz and his co-workers have recently published a three-dimensional electron-density map at 5.5 Å resolution which shows a fascinating combination of simplicity in principle and complexity in detail (109).

Unfortunately, these are the only two globular proteins for which direct methods of x-ray analysis have been successfully applied. For both insulin (110) and ribonuclease (111, 112) the complete sequence of amino acids is available, and this information has led to some ingenious proposals for their configurations (36, 113); but, although both proteins have been studied by x-ray diffraction methods for some years (114 to 117), no direct structure-determination has yet been reported. Accounts of preliminary work on chymotrypsinogen (118), β -lactoglobulin (119), glucagon (120), and ferritin (121) have appeared since the last review.

Method of isomorphous replacement.—The difficulty in extending to other proteins the methods that have been used with hemoglobin and myoglobin is a difficulty in the field of protein chemistry, not of crystallography. The results so far have all come from applying the isomorphous replacement technique to the problem of determining the phase for every reflection in the crystal's diffraction pattern. For this method it is necessary to have, in addition to the natural protein, a number of derivatives which contain atoms of high scattering power for x-rays, such as mercury, lead, or, toward the lower limit of atomic number, iodine. The heavy atom usually "replaces" some of the bound water on the surface or in the interior of the protein molecule. The further requirement that these derivatives should be crystallizable

means that they must be prepared by methods that are quite specific for the selected binding sites on the protein molecule, for any inhomogeneity in surface properties among the molecules is likely to interfere with the crystallization. It is also necessary that the crystal form of any derivative be isomorphous with that of the natural protein; i.e., it should not be changed in any way other than by addition of the heavy atoms. A diagram illustrating some features of this technique is presented in Figure 1. Up to the present time

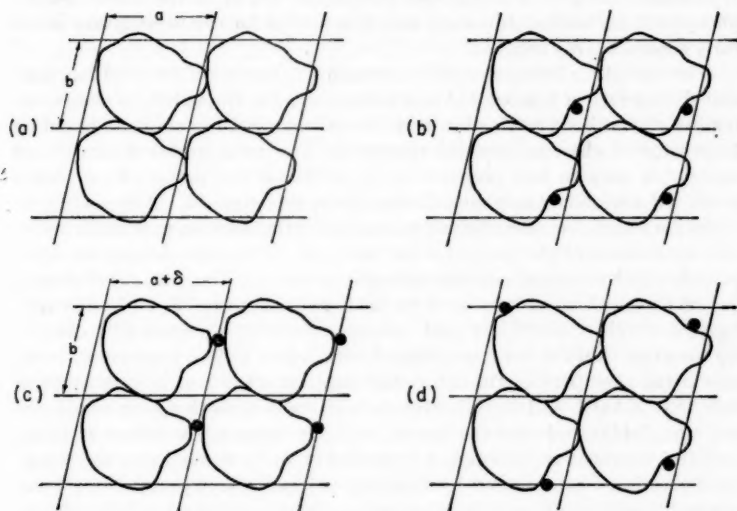


FIG. 1. The isomorphous replacement method. (a) Native crystal with one molecule in the unit cell. (b) Isomorphous replacement where the black dot indicates the heavy atom. (c) Non-isomorphous replacement: a change in one cell dimension. (d) Random replacement: no change in cell dimensions, but the heavy atom does not have a fixed position relative to the protein.

very few reactions have been developed by means of which a limited number of sites can be picked out and labelled in a suitable way. Among the few possibilities, the blocking of sulphhydryl groups with mercurials has been studied most, and this was indeed the first method to provide a derivative suitable for x-ray work (122). However, not all proteins have accessible sulphhydryl groups and each one presents its own special problems.

For enzymes there is the attractive opportunity to block the reactive site with an inhibitor or substrate analogue containing a heavy atom, but complete success has not yet been reported with this approach. King (123) has attempted it with ribonuclease, but the substrates of this enzyme are inconveniently complex. Apparently, mercurated cytidylic acid is bound, and

some oxygenated ions that are multiply charged and act as competitive inhibitors may permit the preparation of chelated heavy metal complexes which can also be bound to the protein. With myoglobin, where the prosthetic heme group might be regarded as the "active site," many attempts have been made to exploit its affinity for imidazoles, isocyanides, and nitroso compounds (107). None of these efforts has produced an entirely satisfactory derivative because these rather weakly bound ligands are displaced by traces of atmospheric oxygen, although one compound linked to the heme, *p*-iodophenylhydroxylamine, did prove useful as a label for the heme group in the early stages of x-ray analysis.

Fortunately, a less systematic approach was successful in providing suitable derivatives of hemoglobin and myoglobin, but the failure of similar attempts with other proteins has emphasized the need for methods based on knowledge of the fundamental chemistry. The most useful derivatives of myoglobin were in fact prepared by crystallizing the protein from nearly saturated ammonium sulphate solution in the presence of a variety of heavy-metal ions; sufficient metallic salt was added to the medium to provide one or two equivalents of the heavy ion per molecule of protein. Among the compounds which produced suitable derivatives and had heavy atoms that were bound at only one or two sites on each molecule were K_2HgI_4 , $KAuCl_4$, $AgNO_3$, $Cl \cdot Hg \cdot C_6H_4 \cdot SO_3H$, and "mercury diamine" (prepared by dissolving mercuric oxide in hot concentrated ammonium sulphate solution). In no case is the chemistry of the interaction understood, but it is interesting to note that $KAuCl_4$ and K_2HgI_4 give derivatives with both sperm whale and seal myoglobins and that the heavy atoms are apparently bound at corresponding points on these different molecules (124). In hemoglobin, too, a useful derivative was obtained by blocking the sulfhydryl groups with iodoacetamide and then crystallizing the protein from ammonium sulphate in the presence of two equivalents of mercuric acetate (125).

X-ray techniques.—In the isomorphous replacement method, the X-ray scattering from the unstained protein crystal is compared with the scattering from suitable crystalline derivatives. The intensities of the various reflections are altered by the presence of the heavy atom, and from these changes it is possible to deduce the phases of the x-ray reflections. This method is described in non-mathematical fashion by Crick & Kendrew (2) and by Green (126). However, the reader will need to refer to original papers for a detailed understanding of the method. The most general description is that by Harker (127), in which he shows that at least two derivatives, isomorphous with the natural protein, are required to determine the phases of the x-ray reflections for any non-centrosymmetric structure. In principle, the use of anomalous dispersion might reduce this requirement (128), but the application to proteins is restricted by the smallness of the scattering factors involved; the situation has been assessed by Blow in his work on hemoglobin (129).

The first task in any three-dimensional analysis is to discover the arrangement of the heavy atoms in the various crystals or, more particularly, the

vector distances between the sites of the various isomorphous replacements. In both horse hemoglobin and sperm whale myoglobin it was possible to obtain two of the three co-ordinates for each heavy atom or group by straightforward difference Patterson methods (107, 122), but the relative separations of the replacements along the third co-ordinate axis had to be determined by more sophisticated correlation techniques (130 to 132a).

The size of the contribution which the heavy atoms make to the diffraction pattern varies from one reflection to another, and in some cases it is too small to provide reliable information about the phase. For this reason, and because of the inevitable random errors in the measurement of the intensities of the reflections, it is generally necessary to have more than the two isomorphous replacements which are required in principle. The most advantageous ways of combining the data from all the replacements, and suitable means of assessing the reliability of the phase determinations, have been discussed by Blow & Crick (133).

It is possible to approach a solution of the protein structure in stages. Thus, the phases can be determined out to a given spacing on the x-ray diffraction pattern, and from these limited data a "low resolution" Fourier synthesis can be made. If the results are encouraging, somewhat more data can then be used to calculate the electron density to a higher resolution. This is of considerable importance in view of the large number of reflections which must be measured. This stepwise technique has been applied on myoglobin and is currently in process in the hemoglobin work. The results obtained in this way are illustrated for diketopiperazine (31) in Figure 2. Individual atoms can be seen at a resolution of 1.5 Å or greater; however, at 2.0 Å the pattern, though blurred, is easy to recognize if one knows what to look for.

Myoglobin.—Myoglobin has a molecular weight of 17,000 and contains some 150 amino acid residues in a single polypeptide chain, together with a prosthetic group, heme, the chemical structure of which is known. Information from the first three-dimensional electron-density map was published by Kendrew and his co-workers in 1958 (135) and was followed by a more detailed account in 1959 (131). This first map at 6 Å resolution was based on phase determinations for the 400 reflections closest to the center of the diffraction pattern; isomorphous derivatives prepared with *p*-chloromercuribenzenesulphonate, aurichloride (AuCl_4), mercury diamine (HgAm_2), and mercuri-iodide (HgI_4) were used. The chosen limit of resolution corresponds to a natural minimum in the mean intensity of the diffraction spectra, which, it was realized, must arise from the presence in the molecule of structures with characteristic dimensions of 10 Å, such as helical polypeptide chains. Such structures would then be resolved in the electron-density map. In fact, the expected type of structure did appear in the form of circular rods of high electron density, sometimes running straight for 30 Å or so but frequently bent into sharp corners, making altogether a compact but irregular bundle (Fig. 3a). These rods revealed the position of the polypeptide chain, with the backbone atoms arranged in a compact form to produce a core of high den-

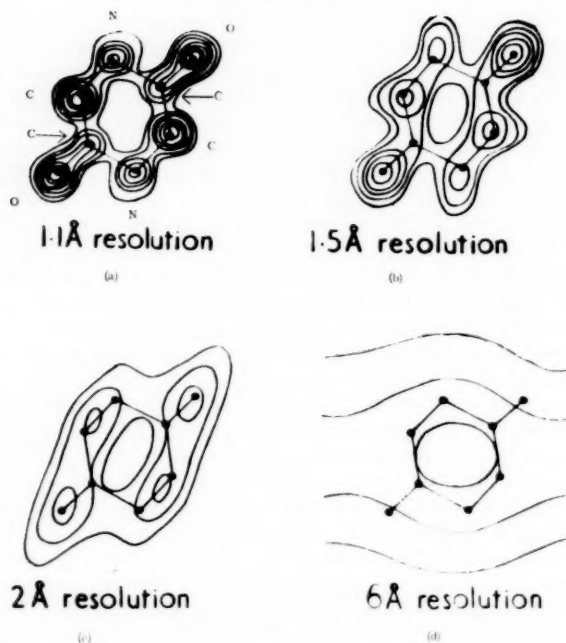


FIG. 2. Electron density map of diketopiperazine calculated with reflections limited at different spacings [reproduced from Hodgkin (134)].

sity, while the side chains were spread out in the lower density regions. The precise nature of the chain configuration could not be determined at this low resolution, and a few discontinuities of the higher density regions made it difficult to trace a unique path for the polypeptide chain through the molecule. The heme group could be identified immediately, for the iron atom produced the highest peak in the electron density, and the difference electron-density map for the derivative with *p*-iodophenyl hydroxylamine revealed the iodine atom in an appropriate place. Since this result was first published, the uncertainties in the continuity of the polypeptide chain have been cleared away, and Kendrew has produced a model which shows clearly the general form of the polypeptide chain and the situation of the heme.

In the next stage, the resolution was taken to 2 Å, a task involving the measurement of 10,000 reflections for the protein and for each of four derivatives (108). To cope with this problem, computing programs were developed to handle these data on a larger electronic computer, the Cambridge EDSAC II (136). From the results, it was possible for the first time to get a detailed impression of the atomic structure of a globular protein. Atoms con-

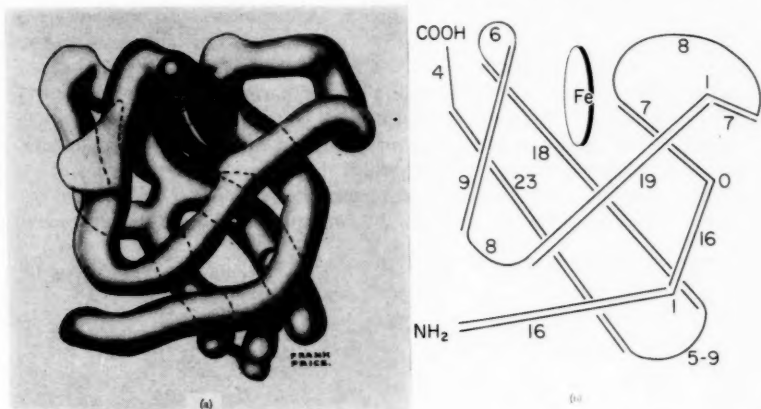


FIG. 3. (a) Tertiary structure of myoglobin deduced from the 6A Fourier synthesis. The heme group is somewhat darker than the rest of the molecule; the plane of the heme group is not correct in this diagram [reproduced from Bodo *et al.* (131)]. (b) Schematic representation of the number of amino acids among the α -helical lengths (double lines) and the "corners" (single lines) as derived from the 2A Fourier synthesis.

nected by covalent bonds are not resolved from one another, but groups of atoms in van der Waals' contact are distinguishable from one another, and, if they are sufficiently large and characteristic, such as the indole group of tryptophan (Fig. 4) or the imidazole ring of histidine, they can be identified.

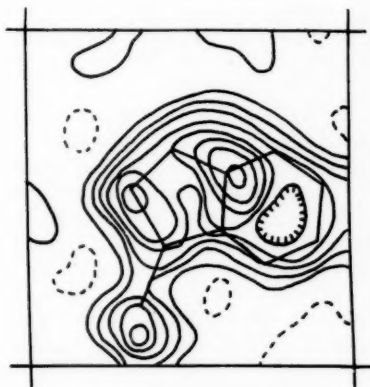


FIG. 4. Electron density contours defining a tryptophan residue in myoglobin in the 2A Fourier synthesis [from J. C. Kendrew (Private communication)].

The rods of high density seen in the 6 Å map now look like tubes, hollow at the center, and with the wall made up from a thread of high density that follows a spiral path with a pitch of 5.4 Å. This pitch is precisely that of the Pauling-Corey α -helix (40), and a detailed comparison confirms the identity most impressively. Figure 5 was constructed from a typical length of one of these tubes by projecting the electron density in the wall onto a cylindrical surface at 1.95 Å radius, then cutting this cylinder parallel to the axis and unrolling it. A similar projection of the backbone skeleton of the α -helix was then superimposed in register with the contours of electron density. Since

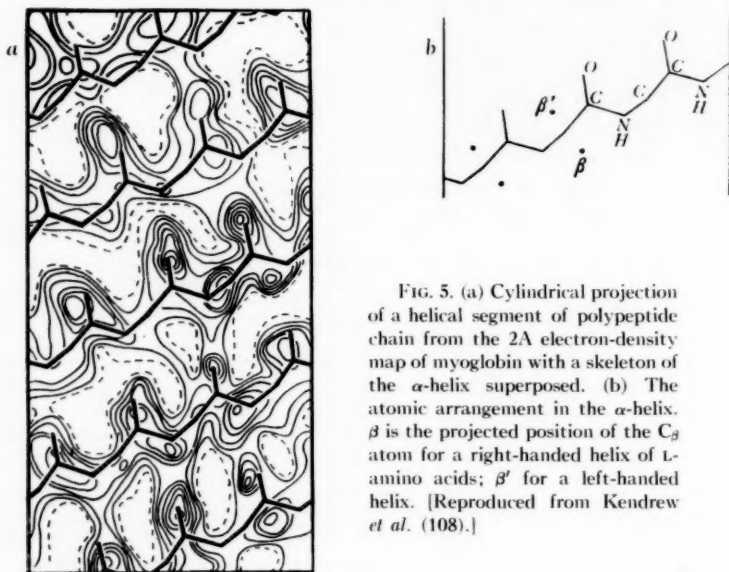


FIG. 5. (a) Cylindrical projection of a helical segment of polypeptide chain from the 2Å electron-density map of myoglobin with a skeleton of the α -helix superposed. (b) The atomic arrangement in the α -helix. β is the projected position of the C β atom for a right-handed helix of L-amino acids; β' for a left-handed helix. [Reproduced from Kendrew *et al.* (108).]

right- and left-handed α -helices differ in the position of C β , it was possible to show that all of the distinct lengths of α -helix in the molecule are right-handed. The sense of the chain is obvious from the orientation of the C—O bonds in the backbone. This has enabled the amino and carboxyl ends to be identified, and the continuous chain of covalent bonding can be traced from one end to the other with only one small region in which there is any uncertainty about the number of residues involved.

The distribution of the amino acid residues among the lengths of α -helix and the intervening regions, where hydrogen bonds are not made in this regular way, is shown in Figure 3b. The figure is schematic, but its general shape is derived from a view of the 6 Å structure shown in Figure 3a. There are three abrupt corners, two with only one residue not hydrogen-bonded into the helix, and one, the corner marked O, which contains a proline residue as

the sole hinge. Proline, not having an amino hydrogen atom, might be expected to determine the formation of "corners" in some way, but chemical analysis shows only four prolines in this type of myoglobin (137), whereas there are seven "corners." Two more prolines have been identified in the electron-density map in longer non-helical sections.

Several features of the heme group and its connection to the protein are also apparent. The orientation of the heme plane relative to the crystal axes agrees closely with measurements made by means of the anisotropy in electron-spin resonance for the iron atom (138), and the distribution of electron

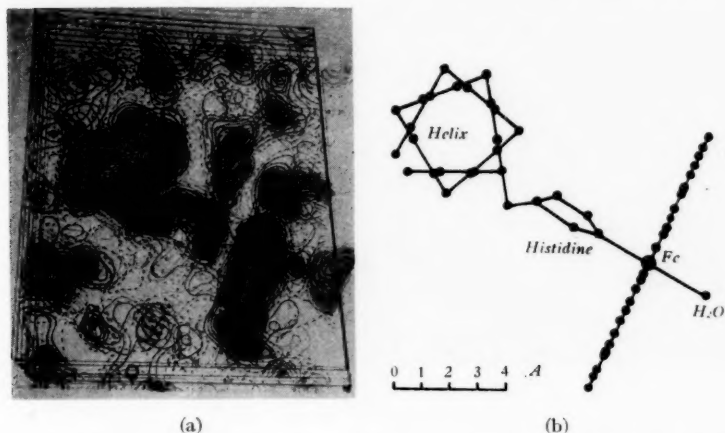


FIG. 6. (a) Photograph of a set of sections normal to the plane of the heme group which shows, from left to right, a helix in cross-section, the histidine residue nearly edge-on, the heme group edge-on, and a presumed water molecule. (b) Sketch showing the atomic arrangement in (a). [Reproduced from Kendrew *et al.* (108).]

density within this plane is very similar to that calculated from the known atomic arrangement seen at 2 Å resolution. As had long been believed on chemical grounds, the iron atom appears to be linked to the protein by co-ordination with a nitrogen atom of a histidine side chain, whereas the sixth co-ordination position, presumably occupied by the oxygen molecule in oxymyoglobin, is filled, in metmyoglobin, by a water molecule (Figure 6). Further links to the protein appear to be made through hydrogen bonds from the side chains of the heme.

A good impression of the detail in which the structure of this molecule has been revealed is given by the photograph of a skeletal model reproduced in Figure 7. This model includes the atoms of the backbone polypeptide sequence, and all of the side chains which had been identified by mid-1960. Chemical analysis of the amino acid sequence is proceeding simultaneously, and a very large measure of correlation has already been established between

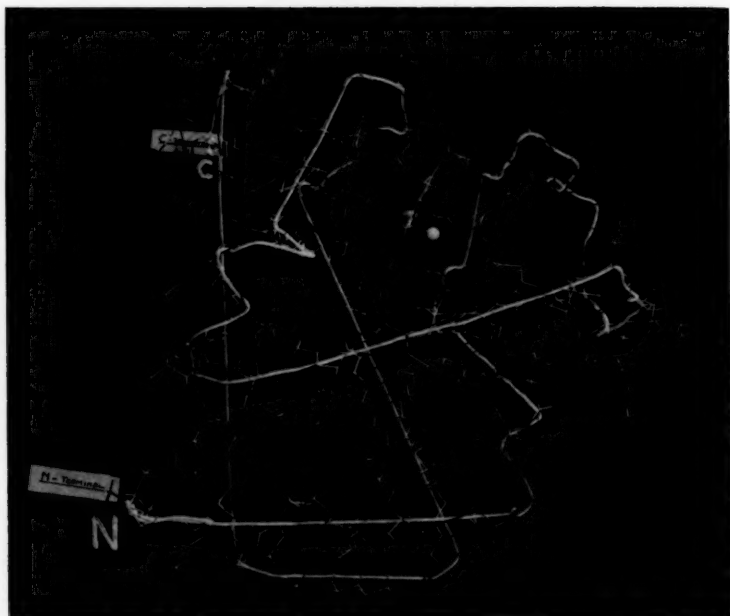


FIG. 7. Photograph of a skeletal model showing the atomic positions in myoglobin as determined from the 2A-resolution electron-density map. A white cord follows the helical segments and polypeptide backbone. N designates N-terminal; C designates C-terminal. [From J. C. Kendrew (Private communication).]

the peptides and the electron-density map. Also, a further stage of the x-ray analysis is being undertaken to incorporate the remaining measurable reflections of the diffraction pattern, which extend out to 1.5 Å spacing (139).

This detailed analysis was carried out with crystals of myoglobin from the sperm whale. Scouloudi (124) has shown that the main chain configuration is similar in seal myoglobin, which produces crystals of a different form. Isomorphous replacement was used to obtain one projection of the electron density in the unit cell, and this was compared with a similar projection of the molecular electron density taken from the three-dimensional map of sperm whale myoglobin. The task of orienting the sperm whale molecule in the seal unit cell was greatly helped by electron-spin resonance measurements of the heme orientation in both crystal forms (138).

Hemoglobin.—Hemoglobin is four times the size of myoglobin, and has four heme prosthetic groups, each of which binds reversibly one molecule of oxygen. Chemically, normal adult human hemoglobin is known to be composed of four polypeptide chains of roughly equal size, but only of two types, one pair being characterized by the N-terminal sequence valyl-leucyl- (α

chain) and the other pair by the sequence valyl-histidyl-leucyl- (β chain) (140). For horse hemoglobin, which has been the material chiefly used in the crystallographic studies, the two chains have the N-terminal sequences valyl-leucyl- and valyl-glutamyl- (141). Several crystal forms of hemoglobin from different species of mammals have shown a symmetry which requires that the molecule be made up of two identical halves, and it seems likely that this is a general feature of mammalian hemoglobins.

The sequence of the first 31 residues from the N-terminus of the β chain of normal human hemoglobin has recently been published by Braunitzer *et al.* (142). This portion includes those amino acids at which specific and genetically determined exchanges take place in some of the pathological hemo-

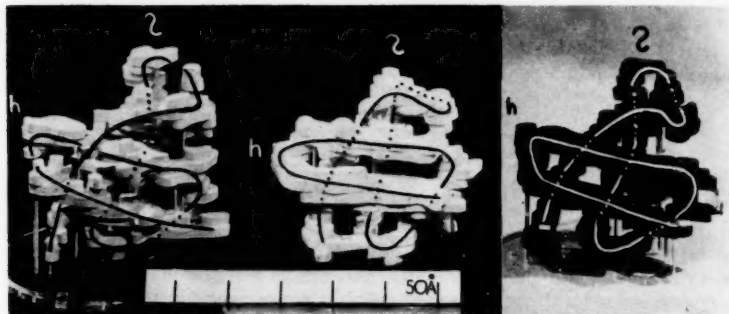


FIG. 8. A comparison of the two different polypeptide chains of hemoglobin with that of myoglobin (right). The heme groups are at the back of the chains. [Reproduced from Perutz *et al.* (109).]

globins—for example, the replacement of a glutamic acid residue by one of valine in sickle-cell hemoglobin (143).

The first results obtained from isomorphous replacement in the monoclinic crystal form of horse hemoglobin were projections of the electron density. These were largely uninterpretable because of overlapping attributable to the great depth of the unit cell. Perutz and his co-workers have now achieved a three-dimensional phase determination, by isomorphous replacement, for all the reflections within a limiting sphere at 5.5 Å spacing; this gives a map in which the detail is comparable with the 6 Å map of myoglobin (109).

The four polypeptide chains are revealed as four distinct subunits arranged tetrahedrally. The subunits must necessarily be identical in pairs because of the symmetry of the crystal, but it has also been found that the two unrelated types of chains (α and β) have similar conformations, and, most significantly, are like the chain in myoglobin (Fig. 8).

This close similarity with myoglobin enables one to draw some conclusions about the hemoglobin molecule by analogy. The sense of the chains must be the same; hence, the amino and carboxyl ends can be identified in

hemoglobin too. Also, the manner of linking the heme to the protein is believed to be similar; thus it is possible to distinguish the reactive side of the heme plane.

The way in which the subunits are assembled is illustrated in Figures 9 and 10. Starting with two chains related by the dyad axis of the crystal (Fig. 9), one may take the other pair at first in the same orientation, then rotate them through a right angle about the dyad, and invert them over the original pair. The resulting arrangement comes close to having the point group symmetry 222, i.e., three dyad axes at right angles to one another. The surfaces of the black and white chains fit in a complementary way over a large area, whereas equivalent chains are hardly in contact at all. Perhaps the most surprising feature is the arrangement of the heme groups, shown diagrammatically in Figure 11.

Hemoglobin binds and releases molecular oxygen in a more complicated way than myoglobin. For myoglobin the oxygen dissociation curve is a simple hyperbola, with a single binding constant as its parameter, but for hemoglobin the curve is sigmoid in shape, a great majority of the molecules being converted between the oxygenated and reduced forms by comparatively small changes in the partial pressure of oxygen around 25 mm. Hg. Effectively, the binding of an oxygen molecule by one heme facilitates the binding of the next molecule, and so on. It was believed that this might be a steric effect, but now that the hemes have been found so far apart (the shortest dis-



FIG. 9. Two pairs of chains from hemoglobin, symmetrically related by the dyad axis. The arrow shows how one pair is placed over the other to assemble the complete molecule [reproduced from Perutz *et al.* (109)].

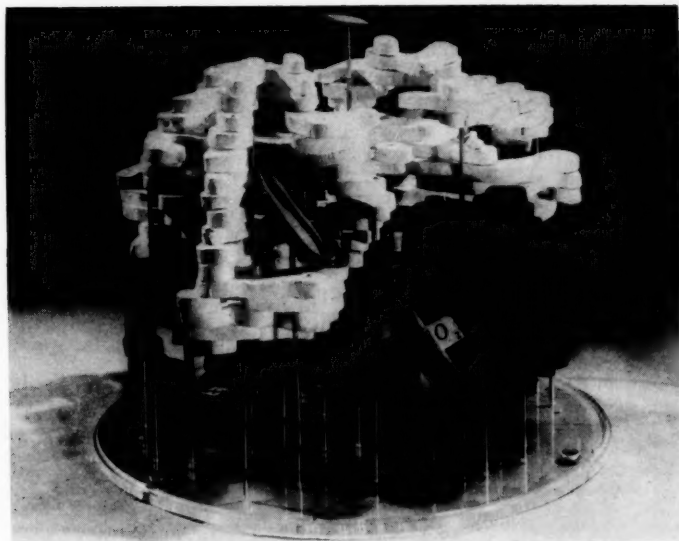


FIG. 10. The hemoglobin model completely assembled. Heme groups are shaded grey, and the position of O_2 molecules is indicated [reproduced from Perutz *et al* (109)].

tance between iron atoms is 25.2 Å, whereas the symmetry-related pairs have separations of 33.4 and 36.0 Å), a more subtle explanation is required.

The observation that the heme-heme interaction is reduced by blocking the sulfhydryl groups with mercurials (144) is of great interest, for the positions of the sulfhydryls are clearly marked by heavy atoms. As shown in Figure 11, they lie between two symmetrically related hemes (on the black chains), with S—Fe distances of about 13 Å and 21 Å, and close to the points of contact between the black and white chains. The spacial arrangement is suggestive of a long path of interaction through the heme-linked histidine and the cystine on the black chain to the white chain (109), but details of this must await an electron-density map at higher resolution.

Even at the present time, when the work is not complete, the x-ray analyses of hemoglobin and myoglobin point the way to answering many questions that concern the synthesis and activity of globular proteins. In time, with further examples of such structure determinations, one may hope to see which features of the polypeptide chain conformation are essential to activity in metabolism and to what extent they are determined by the sequence of amino acid residues.

NUCLEIC ACID COMPONENTS

Rapid progress has been made in the last four years in determining the

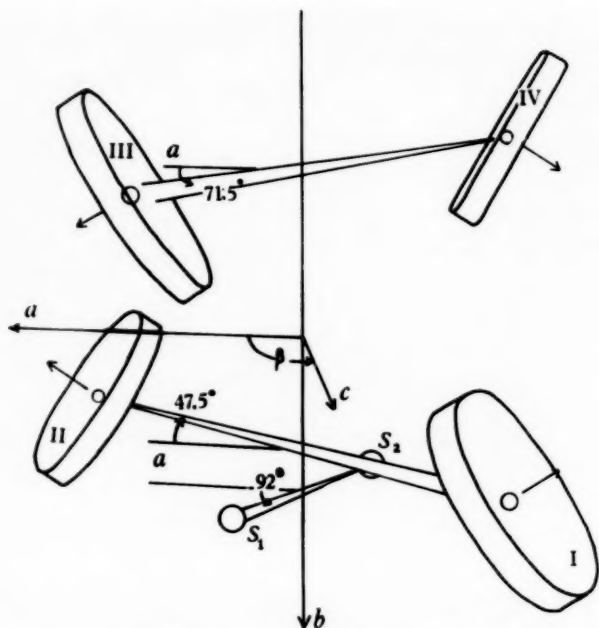


FIG. 11. Arrangement of heme groups in hemoglobin; arrows indicate the reactive side of each heme [reproduced from Perutz *et al.* (109)].

molecular structure of nucleic acid components and related substances. In Table II are listed the structures that have been completed since the previous review by Kendrew & Perutz. This list includes several purine and pyrimidine derivatives, a nucleoside, and three of the naturally occurring nucleotides. An accurate knowledge of the dimensions of these molecules is necessary in order to learn more about the configurational potentialities of the nucleic acids themselves. These provide us with information about tautomeric states, resonance structures, and hydrogen bonding. This is of great value in determining the structure of the more complicated nucleic acid polymers, and, what is equally important, this information is of assistance in showing that certain configurations are impossible. In addition to the structures which have been completed, preliminary unit cell and space group determinations have been made on several purine and pyrimidine derivatives (160, 161), various polymorphic forms of cytidylic acid (162), and on the disodium salt of adenosine triphosphate (163).

Useful summaries of the existing data on purines and pyrimidines, as well as their hydrogen-bonding, have been published by Pauling & Corey (164) and Spencer (165, 166). The conclusion drawn from these studies is that the

TABLE II
STRUCTURES OF NUCLEIC ACID COMPONENTS AND RELATED SUBSTANCES

Compound	Data	Maximum Error*	Remarks	References
Pyrimidine	3-Dimensional and least squares analysis	0.02 Å	Accurate determination	145
2,5-Diamino-4-mercapto-6-methyl pyrimidine	Fourier projections on two planes	0.09 Å	—	146
Theophylline	Generalized projections using 4 crystal planes	0.03 Å	Molecules are linked in pairs by two N-H ··· O hydrogen bonds	147
1,3,7,9-Tetramethyl uric acid	Fourier projection on two crystal planes	Preliminary report	Two polymorphic forms solved with molecules parallel to each other, 3.3 and 3.5 Å apart	148
Caffeine	Fourier projection using two crystal planes	0.06 Å	Discussion of purine bond distances	149 150
1-Methyl thymine plus 9-methyl adenine	—	Preliminary report	A hydrogen-bonded purine-pyrimidine pair	151
Sodium pyrophosphate decahydrate	3-Dimensional Fourier methods	0.18 Å	Angle to the central oxygen P-O-P is 134°	152
Sodium triphosphate	Fourier projections on 3 planes	0.09 Å	Low and high temperature phases studied	153 154
Urea phosphate	Fourier projections on two planes	Preliminary report	—	155
5'-Bromo-5'-deoxythymidine	Fourier projections on two planes	0.3 Å	Discussion of tautomeric forms	156
Cytidylic acid b	Fourier projection on two planes	0.06 Å	Puckering of ribose ring differs from that seen in cytidine	157
Calcium thymidylate	3-Dimensional Fourier and least squares analysis	0.2 Å	Thymine residues are parallel to each other, 3.43 Å apart	158
Adenosine-5'-phosphate	3-Dimensional Fourier data	Preliminary report	Ribose ring puckered with C _{5'} out of plane	159

* The Maximum Error is three times the standard deviation of atomic co-ordinates.

guanine-cytosine base pair in the Watson-Crick structure for DNA is actually held together by three hydrogen bonds instead of two. In addition, Spencer discusses the configuration of the furanose ring in deoxyribose and surveys the possible configurations of the hydrogen-bonded pairs and the nucleotide backbone in DNA.

It is interesting that many of the purine and pyrimidine derivatives form hydrogen-bonded pairs in the crystalline state. Theophylline (147) crystallizes in pairs held together by two N—H ··· O hydrogen bonds in a configuration which is similar to that seen in adenine hydrochloride (167). The importance of hydrogen bonds in maintaining this kind of a configuration (148) has been questioned, since caffeine molecules pack in the crystal in a manner which is very similar to theophylline (149) even though there are no hydrogen bonds to hold the molecules together. The suggestion has been made that dipole-dipole forces may be of equal importance in maintaining the

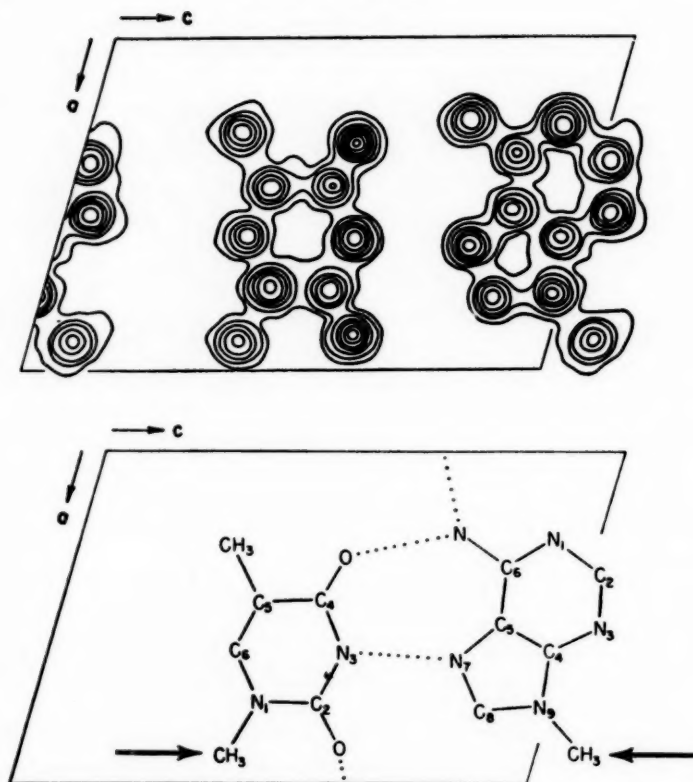


FIG. 12. The electron-density map for the mixed crystal of 1-methyl thymine and 9-methyl adenine; the drawing on the bottom shows the atomic labels, and the heavy arrows point to the carbon atoms which would be part of the sugar molecule in a nucleoside [modified from Hoogsteen (151)].

configuration in the crystal (148). Other examples of hydrogen-bonded pairs of pyrimidines are seen in the crystal structures of 1-methyl thymine (168) and 1-methyl uracil (169). In both crystals, the pairs are held together by two hydrogen bonds between the oxygen on C—2 and the hydrogen on N—3.

A most interesting example of this tendency of purines and pyrimidines to form hydrogen-bonded pairs in crystals is seen in Hoogsteen's mixed crystal of 1-methyl thymine and 9-methyl adenine (151). Figure 12 shows the electron density of both the thymine and adenine derivatives and the manner in which they are hydrogen bonded together. It is most interesting that the hydrogen bonds shown by this complex are not the hydrogen bonds which

have been postulated in the Watson-Crick model of DNA (170). Instead, the thymine is bonded to the adenine amino group and to N—7 of the imidazole ring. This type of hydrogen bonding had been postulated earlier for the three-stranded helix of polyadenylic acid plus two-polyuridylic acid (171); however, the single-crystal investigation provides exact bond angles and distances. This work underlines the fact that there may be other configurational potentialities which can be realized by the nucleic acids and shows the importance of single-crystal studies in supplementing work on the more complex biological materials. It will be of great interest to extend this type of mixed crystal investigation.

NUCLEIC ACIDS AND POLYNUCLEOTIDES

The basic difficulty in working with large fibrous molecules is in obtaining enough experimental x-ray diffraction information to elucidate unambiguously the configuration of the molecule. Quite often one can ascertain some geometrical features; for example, the diameter and pitch of a helical molecule, and even the number of repeating residues in one turn. But often there are not enough data to fix directly the configuration of the repeating unit, and, accordingly, great stress is put upon the construction of a "model" structure and the comparison which can be made between its diffraction pattern and that obtained experimentally. It is important to note that agreement between these two does not mean that the "model" is completely correct; the agreement may be fortuitous or only approximate. However, as more and more data are obtained, the probability of having the correct structure increases. These comments are very well illustrated in the work on the nucleic acids. This subject has been reviewed briefly by Rich (172).

Deoxyribonucleic acid.—Further work has been done on the structure of deoxyribonucleic acid (DNA) by Wilkins and his co-workers (173 to 177). The equilibrium configuration of the DNA molecule in a fiber is very sensitive to water content as well as the type of cation present. Four different configurations of the molecule are known (176), all of which are interconvertable by suitable alterations in relative humidity. In all cases, the molecule exists in the form of a two-stranded helix, but the number of nucleotide pairs per turn of the helix can vary from 9.3 to 11.0 and the base pairs can tilt at various angles to the helix axis. Studies have been carried out on a variety of DNA preparations which contain widely different base compositions and yet all produce the same type of diffraction pattern (173, 175). A detailed study (176) describes the experimental technique used in obtaining high-resolution x-ray diffraction patterns from DNA and the methods for recording the results. Another paper by Wilkins, Langridge, and co-workers (177) deals with a comparison between the diffraction pattern produced by a lithium DNA salt and the calculated diffraction pattern obtained by the two-stranded helical model. The orientation of the molecules has been fixed in the unit cell by utilizing the three-dimensional intensity data. The final configuration adopted is similar in a general way to that suggested by Watson & Crick (178); a major difference is the position of the helix axis which is closer to the

center of the hydrogen-bonding base pair in this model. The final model is tightly packed and has two close contacts of 2.9 Å between carbon and oxygen atoms. The deoxyribose ring is puckered with the C₂ atom 0.3 Å out of the plane. Considerable attention is devoted to the manner in which the DNA helices pack together in the unit cell, since the helix axes are closer together than the diameter of one molecule. This is accomplished by displacing one molecule along its helix axis relative to its neighbor, thereby enabling the molecules to interpenetrate into the helical grooves.

Ribonucleic acid and synthetic polynucleotides.—No additional x-ray diffraction results have been reported for ribonucleic acid (RNA) since the last review (1). However, there have been several new x-ray diffraction studies on synthetic polyribonucleotides by Rich, Davies and co-workers, many of which were carried out in the hope that the results would have relevance to the naturally occurring nucleic acids. All of these synthetic polynucleotides appear to form helical structures with the bases inside and the charged ribophosphate chains outside. Polyriboadenylic acid will form by itself a two-stranded helical complex in which there are eight residues per turn in each helical strand with both strands parallel to each other (179, 180). Physicochemical evidence has been presented (181) which suggests that the helix of polyriboadenylic acid is stable only at a low pH when the nucleotides have taken on an additional proton. Several papers (182, 183, 184) discuss aspects of the structure of this polynucleotide both in solution and in solid state. In the alkaline state, it can also form an alternative regular structure (185).

At neutral pH, polyriboadenylic acid and polyuridylic acid will wrap around each other to form a helical complex with a geometry which is similar to that of DNA (186, 187). In a similar manner, polyriboadenylic acid will combine with a single strand of polyinosinic acid to form a 1:1 helical complex, in this case with a pitch which is a few angstroms larger than that seen in DNA (188). In both of these cases, it is believed that the hydrogen bonding is analogous to the hydrogen bonds which are postulated for adenine and thymine in the Watson-Crick model of DNA. An analogue for the guanine-cytosine pair is seen in the combination of polyinosinic acid and polycytidylic acid. These two polynucleotide chains wrap around each other to form a 1:1 complex (189), and this material has crystallized (190) so that the hydrogen bonding can be studied in detail.

An interesting extension of this work is the formation of three-stranded helical complexes. Thus, it is possible to make polyriboadenylic acid plus two polyuridylic acid (191, 192) or polyriboadenylic acid plus two polyinosinic acid (188). Three-stranded helices of this type have not as yet been found among naturally occurring nucleic acids. It is believed that the third polynucleotide strand is attached by hydrogen bonds that involve the imidazole N—7 and the adenine amino group; these form hydrogen bonds with the carbonyl oxygen and ring nitrogen of uracil or hypoxanthine. As mentioned above, hydrogen bonding of this type has been observed in a single-crystal analysis by Hoogsteen (151). Quite a different type of hydrogen bonding is

believed to provide the framework for a three-stranded helical complex which is seen in polyinosinic acid itself (193). On raising the ionic strength of a neutral solution of this polynucleotide, the polymer chains combine with themselves and there is a drop in optical density. Fibers obtained from this material produce a diffraction pattern which has been interpreted in terms of a three-stranded structure in which all ribonucleotide chains are parallel to each other and are organized around a threefold rotation axis with the hypoxanthine derivatives in the center of the molecule held together by cyclical hydrogen bonds.

Other physicochemical techniques play an important part in complementing the use of x-rays in investigating the structure of these materials. Thus, the stoichiometry of the 1:1 or 2:1 complexes can be obtained directly from the drop in optical density which accompanies the formation of the helical complexes (188, 191, 192). In addition, there are changes in the viscosity of the solution as well as other evidence of aggregation. These techniques have been used by themselves without accompanying x-ray diffraction investigations to carry out additional studies. Fresco & Alberts (194) have found that polyriboadenylic acid will combine with polyuridylic acid even when the former contains some randomly placed uridylic acid nucleotides, or the polyuridylic acid chain may contain occasional adenylic acid residues. They have presented evidence which suggests that the interpolated groups are "looped" out of the main helical complex, instead of being hydrogen-bonded in the center. In this way, the structure is able to accommodate non-complementary bases. This feature of polynucleotide behavior has been used in discussing the secondary structure of RNA (195) and will undoubtedly be the subject of additional investigation in the future.

All of the investigations on synthetic polynucleotides described above were carried out with ribonucleotides which were synthesized by use of the polynucleotide phosphorylase enzyme (196). However, the chemical synthesis of short polymers of deoxyribonucleotides by Khorana and his colleagues [see Tener *et al.* (197)] has made possible studies with these substances. Rich (198) has shown that polyriboadenylic acid will form a two-stranded complementary helix with polydeoxyribothymidylic acid. This suggests a mixed type of DNA-RNA helix which may have physiological implications.

There have been several theoretical discussions of polynucleotides, including considerations of hydrogen bonding (199, 200), the effect of rotational potentials (201), and possible types of three-stranded molecules (202, 203).

NUCLEOPROTEINS AND RIBOSOMAL PARTICLES

Nucleoprotamine and nucleohistone.—At the time of the previous review (1), information had been obtained by Wilkins and his co-workers (204) which indicated that the nucleoprotamine diffraction pattern could be explained in terms of a single strand of an almost fully extended protamine polypeptide wrapped around the DNA helix which lies in the narrow groove

in such a manner that the positively charged side chains interact systematically with the negatively charged phosphate groups. Evidence for this was obtained by studying the chromosomes of live sperm heads which produced a diffraction pattern very close to that of DNA.

The nuclei of most cells contain histone in the chromosomes rather than protamine. However, the configuration here is much less clear. Luzzati and his co-workers (205, 206, 207) have carried out several low-angle x-ray diffraction investigations on various nucleohistone solutions and have adduced evidence for three phases, each of which occurs at a different concentration. These phases are each characterized by a series of low-angle diffraction maxima. One of the phases has the same diffraction pattern as that produced by the fowl erythrocyte nucleus: it shows sharp reflections at a 110 Å spacing as well as at the next two higher orders (55 Å and 37 Å). Luzzati and co-workers have concluded that this may be evidence for a lamellar structure in the chromosomes. On the other hand, Wilkins and his co-workers (208, 209) have been studying oriented fibers of nucleohistone and find, in addition to the DNA diffraction pattern, strong equatorial reflections at 30 and 60 Å and some meridional arcs at 35 and 55 Å. They have presented some evidence which suggests that the strong 60 Å reflection may arise from a phospholipid impurity in the nucleohistone preparations and have discussed various possibilities for the structure of the DNA-histone complex. They suggest that the histone may be in the form of an α -helix running at right angles to the DNA chains to produce the meridional reflections at 35 Å. However, since this is very close to the reflection at 37 Å which Luzzati and co-workers have seen (206), it is possible that this represents a higher order of the fundamental 110 Å repeat. In general, most of the structure proposals presented so far must be regarded as tentative, and further work will undoubtedly be necessary before we know the structure of nucleohistone and the details of chromosomal DNA folding. The relation between the DNA and the protein is not at all clear, since not all DNA is associated with protein, as in bacteriophage and bacteria (210, 211).

Ribonucleoprotein particles.—The work which has been done on ribonucleoprotein particles (ribosomes) illustrates the advantages of combining electron-microscope investigations with x-ray diffraction results. The former technique provides a picture of the external morphology of these particles, the diameter of which is approximately 240 Å. At the same time, x-ray diffraction investigations teach us something about the internal organization of the two major components, protein and RNA.

Electron-microscope studies have been carried out by Hall & Slayter (212) and Huxley & Zubay (213). Both of these studies show that the microsome particle is made up of two units: a larger, somewhat cup-shaped unit called "50 s" because of its sedimentation constant and a "30 s" unit which is smaller and flatter and seems to fit into the concavity of the "50 s" unit. The particles can aggregate together to make a larger "110 s" unit. Negative-staining investigations of these units fail to reveal the polyhedral character which is now so typical of spherical viruses. Positive-staining experiments

show a complex internal structure with no evidence for the existence of a protein shell around the nucleic acid core, as has been found with many small spherical viruses.

X-ray diffraction studies have been carried out on ribosomes by Zubay & Wilkins (214) and Klug and co-workers (215, 216). These were studies of unoriented particles which yielded x-ray powder diagrams. The diffraction pattern of the ribosome is strongly dependent on moisture content, since in the dry state it shows predominantly a disordered diffraction pattern which is largely attributable to the protein. However, when the material is hydrated, it has a series of characteristic spacings which are virtually identical with those found in other diagrams of isolated RNA (217, 218). Support for the concept that the RNA in the ribosomes has its bases stacked together is seen in the fact that the polyribonucleotides have the same hypochromicity within the particles as in the isolated RNA (214, 219). These findings have, of course, underlined some severe conundrums in the structure of RNA. It is clear, for example, in the case of the tobacco mosaic virus, that the RNA is held in a configuration quite unlike that seen in the isolated RNA (see Fig. 12). On the other hand, when this RNA is isolated from the virus, it produces an x-ray diffraction pattern which is more or less identical with that found in RNA obtained from a variety of other sources (217), including isolated ribosomal RNA. One is, of course, tempted to think that this configuration may be important if it is found in the ribosomes, which are the active sites of protein synthesis. On the other hand, x-ray diffraction studies of random copolymers of adenylic acid and uridylic acid or random copolymers containing all four of the naturally occurring ribonucleotides also spontaneously assume this same configuration (180) since they have an x-ray powder pattern identical with that of isolated RNA. The problem, in short, is to explain why all of these ribonucleic acid molecules produce similar diffraction patterns even though some of them probably have biologically meaningful nucleotide sequences whereas the others are completely random. The elucidation of ribosomal structure and mode of operation remains one of the major challenges of the next few years.

VIRUSES

In this section we report the results of recent x-ray studies of tobacco mosaic virus (TMV) and several spherical viruses. A very complete summary up to mid-1960 has been given by Klug & Caspar (220); another summary appeared in 1958 (221). Gierer has also reviewed recent work on TMV (222). Accordingly, we shall treat only the principal topics.

Rod-shaped viruses.—For TMV, which is a rod-shaped virus, Franklin, Klug & Holmes (223) had already presented a schematic picture of the gross structure at the time of the last review, and this picture is essentially unmodified. The rod, 3000 Å long, is built up from some 2130 identical protein subunits arranged on a helix of 23 Å pitch, with 49 subunits to every three turns. The existence of a hole 35 to 40 Å in diameter down the center of the rod, first found as a result of isomorphous replacement studies (224, 225),

has since been confirmed by Huxley (226) and Brenner & Horne (227), who succeeded in staining the inside surface with phosphotungstate, thus making the hole visible in the electron microscope. By use of a similar technique, Nixon & Woods (228) were able to demonstrate an axial periodicity between 20 and 25 Å which corresponds to the pitch of the virus helix. The maximum diameter of the rod has been increased slightly from 170 Å to 180 Å as the result of studies of the binding of osmium to TMV, in which the osmium atoms were found at 90 Å radius (221). This work also lends further support to the picture of a grooved outer surface, which permits neighboring particles to intermesh at a spacing of 150 Å in dry gels of TMV (229).

The achievement of two isomorphous replacements, one with lead (224) and one with methyl mercury (225), together with a comparison of TMV and the repolymerized RNA-free A-protein, have shown all the RNA to be at 40 Å radius, apparently embedded between the protein subunits; however, this leaves several possibilities for its three-dimensional arrangement. A direct proof of the configuration will require the determination of the x-ray phases by isomorphous replacement methods, but other studies have made it seem very likely that the RNA is in the form of a single chain in a helical path of 23 Å pitch and 40 Å radius from one end of the rod to the other without any reversal of direction. Since the RNA helix would interact systematically with the protein subunits, this arrangement could provide a regular relationship between RNA and protein if there were an integral number of nucleotides per subunit. The most likely integer on the basis of phosphorus analyses appears to be three (221). In Figure 13, the helical beaded filament represents the RNA strand in TMV. The length of this RNA helix would be 33,000 Å, which is just the length found for the RNA by Hart (230) in electron-microscope studies of TMV rods partially degraded by treatment with detergent. However, a fully extended polynucleotide chain of that molecular weight would be expected to be some 50,000 Å long, so the sugar-phosphate chain appears to be somewhat folded, its configuration probably being determined by interaction with the protein. Comparison of the birefringence of TMV with that of the repolymerized protein (231, 232) indicates that the purine and pyrimidine bases are more nearly parallel than perpendicular to the axis of the TMV rod, and this is in agreement with observations of the ultraviolet dichroism (233, 234).

Radial electron-density distributions have been obtained for three strains of TMV (235) and for cucumber virus CV4 (235, 236). All show the same sort of structural features as the common strain of TMV and have the same external diameter, but the electron density of CV4 appears to be lower in the outer part of the particle. It has been suggested that this may be correlated with the slightly shorter interparticle distance found in dried CV4 (221).

Some preliminary work has been reported by Franklin (237) on the shape of the protein subunit which suggests that the subunit protrudes into the inner side of the viral rod as well as at the outer surface. However, the internal and external protuberances do not occur opposite one another, thereby

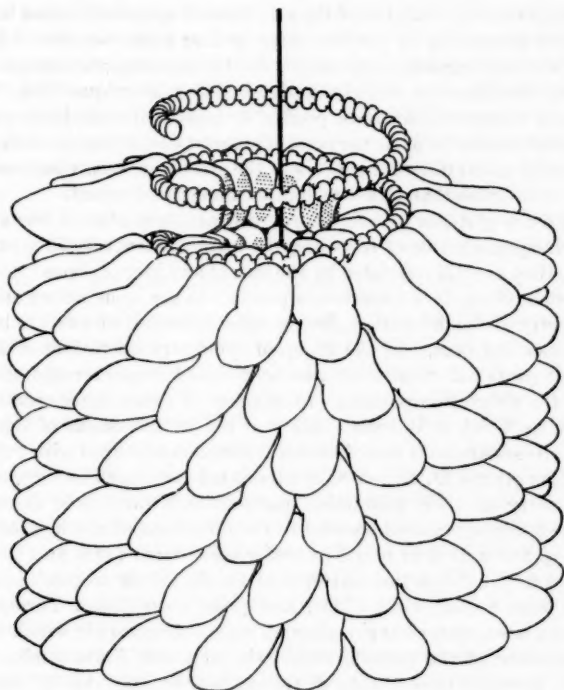


FIG. 13. Diagram of the tobacco mosaic virus; the elongated objects are the protein subunits, and the beads on the helical band represent nucleotides in the RNA [reproduced from Klug & Caspar (220)].

suggesting that the general arrangement of the subunits is not strictly perpendicular to the particle axis. The maximum width of the subunit can be determined by the 22° angle of the wedge-shaped space available for it (238). To a first approximation, the protein subunit can be considered an ellipsoid of revolution with a major axis of 70 Å and a minor axis of about 20 to 25 Å.

One of the more recent developments in the study of TMV has been the complete elucidation of the sequence of amino acids in the protein subunit by Schramm, Fraenkel-Conrat, and their co-workers (239, 240). They have been able to show that cysteine is the twenty-sixth residue from the amino end, and its position in space is fixed by the attachment of methyl mercury (225) which showed up 56 Å from the TMV helix axis. An extension of this type of work to give to various elements of the polypeptide chain assigned positions in space will undoubtedly facilitate elucidation of the complete three-dimensional structure of the protein subunit.

Spherical viruses.—Studies of the structure of spherical viruses have been particularly interesting in the last three or four years because of improvements in the preparation of specimens for the electron microscope. In particular, the development of the negative-staining technique (226, 227, 241) has brought results which can be profitably compared with deductions from the x-ray diffraction data. At the present stage of development, these two approaches still reveal different aspects of the structure, but, when reconciled, they add up to more than either technique alone could reveal.

As with the globular proteins, x-ray diffraction studies of virus crystals are, in principle, capable of revealing the internal structure in great detail. However, they are still restricted by the lack of any isomorphous replacement in a spherical virus. It is nonetheless possible to get some information from the symmetry of the diffraction photographs. Spherical viruses are built to a very symmetrical plan, and the group of symmetry operations relating the component parts one to another can be deduced from corresponding symmetry in the diffraction pattern, independent of phase determination. The suggestion by Crick & Watson (242) that the protein shells of the smaller spherical viruses are built out of identical subunits arranged with cubic symmetry (point groups 23, 432, or 532) stimulated the search for suggestions of this symmetry in their diffraction patterns. Characteristic clumping of stronger reflections oriented parallel to the directions of the fivefold axes in point group 532 were first found in bushy stunt virus (243) and have since been found in the diffraction patterns of turnip yellow mosaic virus (244), Southern bean mosaic virus (245), and polio virus (246). Turnip yellow mosaic virus has a somewhat complicated crystal structure in which there are two orientations of the particle within the unit cell. Fortunately, this arrangement provides opportunity of testing how strictly the 532 symmetry applies to the virus, for, if it is rigorously obeyed, certain reflections in the diffraction pattern should have strictly zero intensity (244). With crystals of the complete virus, Klug & Finch found that these reflections are not zero, but for crystals of the "top component," which is the protein shell free of nucleic acid, all the appropriate reflections with spacings greater than 35 Å are certainly zero (247). It appears that most, if not all, of the protein shell is arranged with 532 symmetry, whereas the nucleic acid has the lower tetrahedral symmetry of point group 23, required by the crystal structure as a whole. Any object with 532 symmetry is geometrically divisible into 60 identical subunits, and 23 symmetry requires 12 subunits, but as yet the x-ray studies give no information about the shape of these units or their chemical nature.

Recent electron-microscope studies of turnip yellow mosaic virus (248, 249) have identified 32 "lumps" which constitute the surface shell of each particle; these morphological subunits are arranged at the vertices of a semi-regular polyhedron that has 532 symmetry. Whatever the precise form of the polyhedron, the 32 vertices must be of two distinct types: 12 at the ends of fivefold axes and 20 at the ends of threefold axes; hence, the number of

geometrical subunits is at least 120. The problem of identifying these with chemical subunits, i.e., the polypeptide chains, has not been solved. The simplest alternatives appear to be either that there are two distinct chemical species of subunit or that the same unit aggregates in two different ways to produce the different types of morphological unit. If this second alternative is correct, it would seem likely that the two types of "lumps," which are indistinguishable by size or shape in the electron microscope, are made up from sets of five and six chemical units, rather than five and three, to make 180 chemical subunits in all. This all points to the need for chemical analysis to supplement the deductions from x-ray and electron-microscope studies.

CARBOHYDRATES AND OTHER COMPOUNDS

In the period under review, the conformations of three more simple sugars, β -D-arabinose (250), α -L-rhamnose (251), and β -D-glucose (252), have been revealed in crystal structures, and a preliminary report on the disaccharide cellobiose has also been published (253). All these provide further examples of the pyranose ring in the Sachse "chair" form. It is particularly interesting to see that in all the relevant crystal structures worked out so far, i.e., chitosamine (254), α -glucose (255), sucrose (256), β -glucose, and cellobiose, the glucose unit shows only one of the two possible "chair" conformations, with the non-hydrogen groups on C₂, C₃, C₄, and C₅ arranged equatorially. In cellobiose the relative orientation of the two 1,4- β -linked glucose rings is different from that which has been assumed in the postulated structure of regenerated cellulose (257), but this may only be a special feature of this particular crystal structure.

Among compounds which are metabolically related to the sugars, we may first mention a group of studies by Patterson and co-workers on the substrates of aconitase. In the course of the degradation of acetate via the citric acid cycle, aconitase catalyzes the establishment of equilibrium between citrate, *cis*-aconitate, and *d*-isocitrate. Although citrate is optically inactive, only one of the four possible optical isomers of isocitrate, the dextro, is produced from it. In elucidating the geometry of this interconversion, crystal structures have been worked out for rubidium dihydrogen citrate (258), anhydrous citric acid (259), and the lactone of natural isocitric acid, isolated from leaves of *Bryophyllum calycinum* (260).

The configuration found for the citrate ion agrees with the general rule that in α -hydroxy carboxylic acids the carboxyl group is oriented so that the hydroxyl group comes close to the carboxyl plane. Several examples of this have been discussed by Jeffrey & Parry (261), and further examples have since been found in the gluconate ion (262) and ammonium hydrogen D-tartrate (263). The crystal structure of the lactone confirms the conclusion of Gawron & Glaid (264), from chemical studies, that neighboring carboxyl groups are *cis* with respect to the lactone ring; thus, if the α -carbon atom has the L-configuration, as concluded by Greenstein *et al.* (265), the configuration of natural isocitric acid is L₈. It is proposed (260), in any case, to determine

the absolute configuration of isocitrate in the rubidium salt by making use of the anomalous scattering of Zr K_{α} x-rays by rubidium, as was done by Bijvoet *et al.* for Na-Rb-tartarate (266).

In this group of compounds we may also mention the crystal structure determination for malonic acid (267) and a detailed refinement of the structure determination for succinic acid (268). Crystal structures have now been determined for the dicarboxylic acids $\text{HOOC}(\text{CH}_2)_n\text{COOH}$ with n equalling 1, 2, 3, 4, 5, and 8 (267). In all of these, except malonic acid ($n=1$), the molecule is essentially planar, the even-numbered members of the series being centro-symmetrical about the central C—C bond, whereas the odd-numbered members have a twofold axis through the central C-atom. This usual scheme, applied to malonic acid, would bring the carboxyl groups too close, and it has been found that one of them is rotated out of the plane through 90° to avoid this steric interference.

In the field of polysaccharides, Carlstrom (269) has developed a model for the structure of α -chitin, which makes up the major part of the skeletal and connective tissues of invertebrates. This model has some similarity to the commonly accepted model for cellulose (270, 271). X-ray work has also been reported on hyaluronic acid (272), but the patterns show no orientation.

Many moderately complex molecules have been analyzed by x-ray diffraction methods in the field of natural products. This is in large part attributable to the wide availability of fast electronic computers. The x-ray structures of three elaborate alkaloids, annotinine (273), aspidospermine (274), and ibogaine (275), have been announced almost simultaneously with similar results from chemical degradation studies. In the case of the simpler alkaloid muscarine (276), the primary structural evidence is from x-ray analysis confirmed by x-ray identification of some of the degradation products (277).

Faced with the diversity of other biologically interesting compounds, the crystal structures of which have been determined recently, we can do no more than mention choline chloride (278) and acetylcholine bromide (279); the antibiotics aureomycin (280), terramycin (281), and morellin (282); the narcotics methadone (283) and alphaprodine (284); carcinogens 9,10-dihydro-1,2,5,6-dibenzanthrene (285) and 9,10-dimethyl-1,2-benzanthrene (286); and two porphyrin derivatives, nickle etioporphyrin (287) and phyllochlorinester (288).

The structure determination for vitamin B_{12} , which marked such a sudden expansion in the scope of the technique, was described in the last of these reports, and a more detailed account has since appeared (3). Refinement of the analysis for the hexacarboxylic acid fragment of B_{12} has now probably reached the limits of accuracy of the x-ray data and, taken in conjunction with the chemical evidence, leaves very little uncertainty in the identification of all the 67 atoms in this molecule (289).

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CHEMISTRY OF THE NUCLEOTIDES¹

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Recent articles have dealt mainly with the biochemical and biological aspects of the nucleic acids, and it is now some five years since Brown & Todd (1) reviewed in particular the chemistry of the subject. A considerable amount of work dealing with the chemical synthesis of nucleosides, nucleotides, nucleotide anhydrides, and oligonucleotides has been published during this time. This work, and other chemical studies on nucleotides and nucleic acids which have come to hand up to August, 1960, form the subject matter now reviewed. A comprehensive listing of the literature, even within the limitations indicated, has not been attempted.

NUCLEOSIDES

The preparation of nucleosides by condensation of a mercuripurine or -pyrimidine with a suitably protected halogenose has been extended to the synthesis of the naturally occurring 2'-deoxynucleosides. Previous attempts at deoxynucleoside synthesis using 2-deoxyhalogenoses were unsuccessful because of the low stability of the halogenose. However, Ness & Fletcher (2) have avoided extensive dehydrohalogenation by the use of 3,5-di-O-*p*-nitrobenzoyl-2-deoxy-D-ribofuranosyl chloride. Condensation with chloromercuri-6-benzamidopurine followed by deacylation of the product gave a mixture of 2'-deoxyadenosine and the α -anomer. Fox and co-workers [Hoffer *et al.* (3)] have used crystalline 3,5-di-O-*p*-chloro (or *p*-methyl)benzoyl-2-deoxy-D-ribofuranosyl chlorides for the synthesis, in excellent yield, of thymidine and deoxycytidine (together with smaller amounts of the α -anomers) by reaction of the halogenose with suitable mercuriprimidines. The 5-fluoro analogues of deoxyuridine and deoxycytidine have been prepared by the same approach. The synthesis of 2'-deoxyadenosine and 2'-deoxyguanosine by treatment of silver 2,8-dichloroadenine with crude 3,5-di-O-acetyl-2-deoxyribofuranosyl chloride has been reported by Venner (4).

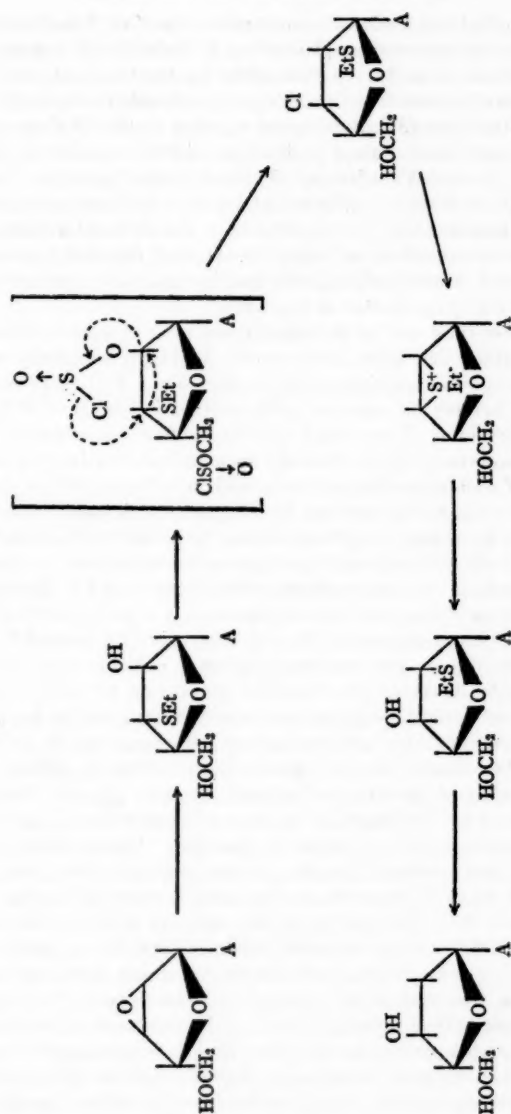
This general method—coupling of a sugar with a preformed base—has now been used extensively, particularly by Baker and his co-workers, for the synthesis of a large number of analogues of the naturally occurring nucleosides. These include 9-glycosyl purines derived from D-glucofuranose (5), L-talofuranose (6), L-rhamnopyranose (7), L-rhamnofuranose (8), 6-deoxyglucofuranose (9), 6-deoxy-L-idofuranose (10), 6-deoxy-D-allofuranose (11), tritiated D-ribofuranose (12), 5-deoxy-D-ribofuranose (13), 5-deoxy-5-fluoro-D-ribofuranose (14), and lactose (15). Various analogues (16, 17) of the aminonucleoside derived from puromycin, including 3'-amino-3'-deoxyadenosine (18), have also been prepared. Considerable variation of the purine moiety is also possible (19 to 23); Davoll (24) has described the syn-

¹ The survey of the literature pertaining to this review was concluded in August, 1960.

thesis of the 8-aza analogues of adenosine, guanosine, inosine, and xanthosine from appropriate triazolo pyrimidines. The general method, first extended to the synthesis of pyrimidine nucleosides by Fox and co-workers (25), has also been applied with considerable success to the synthesis of a number of N³-glycosyl pyrimidines, including 3'- and 5'-amino nucleosides (26) and 5'-fluoro nucleosides (14), in addition to the naturally occurring nucleosides (27). In the main, the rule proposed by Baker and co-workers in 1954 (28), stating that in this type of synthesis the stereochemistry of the glycosyl linkage is controlled by the 2-acyloxy group of the sugar, resulting in formation of a nucleoside containing a C¹-C² *trans* configuration regardless of the original configuration at C¹-C² of the sugar, has been shown to have a wide validity and, indeed, is now used as a proof of configuration. Since the directive effect of a 2-acyloxy group is absent in a ribofuranosyl halide carrying a 2,3-cyclic carbonate-protecting group (and in 2-deoxy-D-ribose derivatives), both α and β forms of the nucleoside can be obtained in these cases. Thus, Wright and co-workers (29) found that condensation of 5-O-benzoyl-D-ribofuranosyl bromide-2,3-cyclic carbonate with chloromercuri-6-benzamido-purine followed by removal of the protecting groups yielded both adenosine and the anomeric 9- α -D-ribofuranosyl adenine.

Since the direct route to 9-D-arabinofuranosyl adenine from arabinohalogenose and metallopurine gives the α -anomer, the β -nucleoside must be prepared by an alternative approach. One such approach has been used by Lee *et al.* (30). Methane sulphonylation of 9-(3',5'-O-isopropylidene- β -D-xylofuranosyl)-adenine followed by removal of the isopropylidene group gave 2'-O-methanesulphonyl nucleoside which on treatment with base gave 9-(2',3'-anhydro- β -D-lyxofuranosyl)-adenine. Treatment of the anhydro nucleoside with the powerful nucleophilic reagent, sodium benzoate in wet dimethyl formamide, then gave a good yield of 9- β -D-arabinofuranosyl-adenine with only a trace of the xylofuranosyl derivative.

This example illustrates the use of epoxides for conversion of a xylose configuration to an arabinose; ring opening is by nucleophilic attack at C³ of the sugar rather than the C² position. Such a route is thus of value for the synthesis of 3'-deoxynucleosides but not for the naturally occurring 2'-deoxynucleosides. Anderson, Goodman & Baker (31), however, have applied related methods, which depend on the selectivity of nucleophilic attack on a nucleoside-2',3'-episulphonium ion to effect an ethylthio migration from C^{3'} to C^{2'}, for the synthesis of 2'-deoxyadenosine. Chloromercuri-6-benzamidopurine was condensed with 2-O-acetyl-5-O-methoxycarbonyl-3-O-*p*-toluenesulphonyl-D-xylofuranosyl chloride, and the product was treated with base to give 6-amino-9-(2',3'-anhydro- β -D-ribofuranosyl)-purine, which with sodium ethylmercaptide yielded 6-amino-9-(3'-deoxy-3'-ethylthio- β -D-xylofuranosyl)-purine. Successive treatments with thionyl chloride and sodium bicarbonate gave 6-amino-9-(3'-chloro-2',3'-dideoxy-2'-ethylthio- β -D-arabinofuranosyl)-purine. "Acetolysis" of this chloronucleoside then gave 6-amino-9-(2'-deoxy-2'-ethylthio- β -D-arabinofuranosyl)-purine, which on desulphurisation yielded 2'-deoxyadenosine (I).

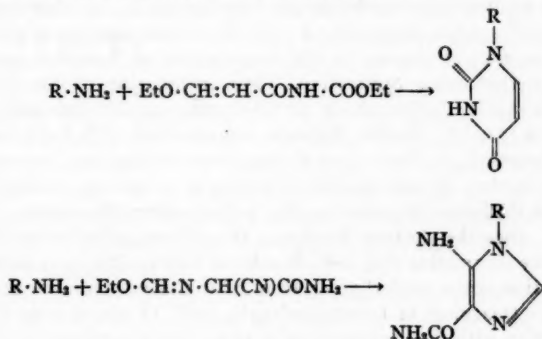


In the pyrimidine nucleosides, inversion of a 2' or 3' hydroxyl is readily achieved via cyclonucleosides [Michelson & Todd (31a)]. A general method for the synthesis of such cyclonucleosides by treatment of the 2' (or 3' or 5')-O-sulphonylnucleoside with sodium-*tert*-butoxide has been developed by Letters & Michelson (32). Subsequent opening of the O²,2'-cyclonucleoside gives the β -D-arabinofuranosyl pyrimidine, as in the syntheses of spongouridine [Brown, Todd & Varadarajan (33)] and spongothymidine [Fox, Yung & Bendich (34)], and 3- β -D-lyxofuranosyl thymine has been prepared from the xylosyl pyrimidine (35). The versatility of 2',3',5'-tri-O-methanesulphonyl uridine as an intermediate for the synthesis of all four 3- β -D-pentofuranosyl uracils (ribosyl, arabinosyl, xylosyl, and lyxosyl) has been amply demonstrated by Codington, Fecher & Fox (36).

Apart from their use for the specific inversion of sugar hydroxyls, O²,2'-cyclopyrimidine nucleosides have proved strikingly successful as intermediates in the synthesis of 2'-deoxynucleosides. Thus, 5'-O-acetyl-2-O-toluene-*p*-sulphonyl uridine on reaction with sodium iodide gave 5'-O-acetyl-2'-deoxy-2'-iodouridine, from which 2'-deoxyuridine was obtained by reduction and deacetylation (37). Since the same iodo derivative was obtained by the action of sodium iodide and acetic acid on 5'-O-acetyl-O²,2'-cyclopyrimidine (to which it could be reconverted by treatment with base), Brown, Parihar & Todd (38) consider the cyclonucleoside to be an intermediate in the replacement of the toluene-*p*-sulphonyl group by iodination, the final product being the result of two displacements with inversion at C^{2'}. The same procedures applied to 5'-O-acetyl-3- β -D-ribofuranosyl thymine yielded thymidine (37). In an alternate approach, Shaw & Warrener (39) treated 5'-O-trityl-5-methyl-2-thiouridine with methanesulphonyl chloride and obtained the cyclonucleoside directly. (Nucleophilic attack at C^{2'} with expulsion of sulphonate ion would be expected to be even more effective by the thiocarbonyl function than by a carbonyl group.) Ring opening by acidic hydrolysis followed by desulphurisation gave a small yield of thymidine.

Construction of the nucleoside from a simple glycosyl derivative has been developed by Baddiley and co-workers in chemical studies paralleling the biosynthetic route to purine nucleotides. Treatment of 2,3,5-tri-O-benzoyl- β -D-ribofuranosyl chloride with sodium azide gave the glycosyl- β -azide, which was converted to an anomeric mixture of ribosyl amines by catalytic reduction. The reaction of this mixture with benzyloxycarbonyl-glycyl chloride followed by debenzoylation yielded the α - and β -anomers of N-(benzyloxycarbonylglycyl)-D-ribofuranosyl amine separated by crystallisation. The free N-glycylribofuranosyl amines were then obtained by hydrogenolysis (40). For the synthesis of the ribofuranosyl imidazole intermediates, the silver or mercuri salt of methyl-5-nitroimidazole-4-carboxylate was treated with 2,3,5-tri-O-benzoyl-D-ribofuranosyl chloride and the product was converted to a mixture of nitroamides by means of methanolic ammonia. Catalytic reduction of 1- β -D-ribofuranosyl-5-nitroimidazole-4-carboxamide gave the 5-aminoimidazole-4-carboxamide glycosyl, which was converted to

inosine via the formyl derivative (41). Shaw and co-workers (42) have used the ribofuranosyl amine mixture mentioned for the synthesis of pyrimidine nucleosides. In addition to a number of 5-cyano-3-glycosyl uracils (43) prepared by the reaction of D-glycosylamines with α -cyano- β -ethoxy-N-ethoxycarbonylacrylamide, uridine was synthesised in similar fashion by treatment of β -ethoxy-N-ethoxycarbonylacrylamide with tri-O-benzoyl-D-ribofuranosyl amine followed by deacylation of the product (II) (42). An analogous reaction of tri-O-benzoyl-D-ribofuranosyl amine with β -ethoxyacryloylisothiocyanate or β -methoxy- α -methylacryloylisothiocyanate yielded, respectively, 2-thiouridine (converted to uridine by the action of aqueous chloroacetic acid) and 5-methyl-2-thiouridine (42).



II

In all cases only the β -nucleoside was obtained, and it is likely that pyrimidine nucleoside formation via linear precursors of the type ribose- $\text{NH} \cdot \text{CH} = \text{CR} \cdot \text{CO} \cdot \text{NHCOOEt}$ is controlled by the 1,2-*trans* configuration at the glycosyl linkage. Indeed, models show that β linear forms should cyclise more readily than the α -anomers in which steric hindrance of the NH group by the 2'-O-benzoyl group occurs (42). An alternate synthesis of 5-amino-1- β -D-ribofuranosylimidazole-4-carboxamide (and hence of inosine) employs condensation of the tribenzoyl ribofuranosyl amine with ethyl-N-(carbamoylcyanomethyl)-formimidate (II) (44).

Thiation of the purine and pyrimidine base by treatment with phosphorus pentasulphide has been applied to suitably protected 6-hydroxy nucleosides by Fox and co-workers (45, 46) and provides a simple route to compounds of possible importance as anti-tumour agents, since the thio derivatives are quite reactive and can be converted to a wide range of 6-substituted analogues. Thiation procedures have been applied to the naturally occurring purine ribonucleosides (45) and to both the ribo- and deoxyriboypyrimidine nucleosides (46). The addition of formaldehyde to pyrimidine nucleotides to form 5-hydroxymethyl and 5-methyl derivatives

is of importance in the metabolism of nucleic acids. Cline, Fink & Fink (47) have developed chemical procedures by which reaction of uridine and 2'-deoxyuridine with formaldehyde gave 5-hydroxymethyl derivatives that on hydrogenation yielded 5-methyluridine and thymidine respectively.

Stevens and co-workers (48, 49) have shown that oxidation of adenine, adenosine, and the adenylic acids with hydrogen peroxide gives the respective 1-N-oxides. Alkaline hydrolysis of adenosine-1-N-oxide gave 1- β -D-ribofuranosyl-5-aminoimidazole-4-carboxamidoxime. A similar instability toward alkali is evident when inosine is alkylated at N¹. Thus, Shaw (50, 51) has shown that alkaline hydrolysis of 1-benzylinosine or of N¹-toluene-*p*-sulphonylinosine (51) gives ribosylimidazole derivatives which can be converted to 1-ribosyl-5-aminoimidazole-4-carboxamide. An alternate degradation of certain purine nucleosides by alkali involves cleavage of the imidazole ring, a reaction analogous to the degradation of 9-methyl purine to 5-amino-4-methylamino pyrimidine. Thus, nebularine (9- β -D-ribofuranosyl purine) is rapidly broken down to 4,5-diamino pyrimidine and its ribosyl derivatives (52, 53). Similar cleavage was obtained with 9-glycosyl derivatives of 6-methyl-, 6-chloro-, and 6-methylmercaptapurine, but not with the 6-dimethylamino, 6-methylamino, 6-amino, or 6-hydroxy compounds. It is likely that the initial degradative step is the nucleophilic attack of hydroxyl ion at C⁸, since the electron density at this carbon is dependent on substituents in the pyrimidine ring (54). Brookes & Lawley (55) have demonstrated the migration of the methyl group from N¹ to the extra nuclear amino group on alkaline treatment of 1-methyladenylic acid. This is of some significance in connection with the identification of minor bases in ribonucleic acid.

Catalytic oxidation of ribo- and deoxyribonucleosides gives the corresponding 5'-carboxylic acids. The results indicate a possible procedure for the stepwise degradation of polydeoxynucleotides by simple or decarboxylative elimination of phosphate [Reese *et al.* (56)].

NUCLEOTIDES

Adenosine-3',5'-cyclic phosphate.—Cook, Lipkin & Markham (57) have isolated adenosine-3',5'-cyclic phosphate from a barium hydroxide digest of adenosine-5'-triphosphate. The cyclic phosphate proved to be identical with an adenine nucleotide formed from adenosine-5'-triphosphate by particulate preparations from dog liver, heart, skeletal muscle, or brain (58). Biologically the nucleotide functions as an intermediate agent in the adrenocorticotrophic hormone-induced stimulation of adrenal phosphorylase and also stimulates the production of steroid hormones by the adrenal cortex (59).

Investigations by Lipkin and his co-workers (60) have fully established the structure of the nucleotide. Equilibrium sedimentation in the ultracentrifuge gave the molecular weight of the compound, while the presence of diesterified phosphate, indicated by titration and by the resistance to attack by phosphomonoesterases, was in accord with the electrophoretic, ion exchange, and paper chromatographic characteristics. Treatment of the nucleotide with liquid anhydrous hydrofluoric acid gave ribose, whereas

methylation followed by this treatment yielded a methyl ribose with the properties of 2-O-methylribose. Hydrolysis with barium hydroxide gave a mixture of adenosine-3'- and -5'-phosphate but no -2'-phosphate. Confirmation of the structure was obtained by synthesis of adenosine-3',5'-cyclic phosphate from the nucleoside-5'-phosphate by treatment with dicyclohexylcarbodiimide under appropriate conditions (60).

Methods of phosphorylation.—Pyrimidine ribonucleoside-2',5'- and 3',5'-diphosphates are conveniently prepared by direct treatment of the nucleoside with polyphosphoric acid. As described by Hall & Khorana (61), the reaction produces considerable amounts of O²,2'-cyclonucleoside-3',5'-diphosphates and, hence, arabinosyl derivatives [Walwick, Roberts & Dekker (62)]. This is avoided in the modification described by Michelson (63).

Turner & Khorana (64) have used *p*-nitrophenyl phosphorodichloridate for the phosphorylation of protected nucleosides. Since alkaline treatment of thymidine-3'-*p*-nitrophenyl phosphate gave both the 3'-phosphate and the 5'-phosphate (via an intermediate 3',5'-cyclic phosphate), the *p*-nitrophenyl residue was removed by treatment with alkali before acidic hydrolysis of the 5'-triphenylmethyl-protecting group. Dichlorophosphoric anhydride, described by Grunze & Koransky (65), would appear to be an excellent phosphorylating agent. Treatment of the 2',3'-isopropylidene derivatives of guanosine and adenosine with this reagent gave high yields of the corresponding 5'-phosphates.

Methods of phosphorylation have been developed recently in which the effective phosphorylating agent is probably a monomeric metaphosphate derivative [Todd (66)]. These are readily obtained by treatment of a monoesterified phosphate with the chloride of a strong acid (such as toluene-*p*-sulphonyl chloride) to give an intermediate mixed anhydride, which then yields the strong acid anion and the alkyl or aryl monomeric metaphosphate. Alternatively, treatment of the phosphate monoester with excess dicyclohexylcarbodiimide in pyridine may be employed. Gilham & Tener (67) have demonstrated the powerful nature of this approach to nucleotide synthesis by the phosphorylation in high yield of nucleoside secondary hydroxyls, which are generally somewhat resistant to phosphorylation by milder reagents. A satisfactory reagent is the metaphosphate derived from β -cyanoethyl phosphate, since the β -cyanoethyl group is readily removed by treatment with alkali. The related β -carbamylethyl phosphate would presumably be effective also. Direct esterification of alcohols by the imidoyl phosphate intermediate from trichloroacetonitrile has been claimed, and the synthesis of adenosine-5'-phenyl phosphate by treatment of 2',3'-isopropylideneadenosine with phenyl phosphoric acid and trichloroacetonitrile has been described by Cramer & Weimann (68).

NUCLEIC ACIDS

One of the major problems in the nucleic acid field is the determination of base sequence in high molecular weight polynucleotides. This can scarcely be attempted until reasonably pure preparations of homogeneous nucleic

acids are available. Cellulose anion exchange materials such as epichlorohydrintriethanolamine and diethylaminoethyl cellulose (69) are now being used extensively for the fractionation of biologically active nucleic acids, ribonucleoproteins, and virus preparations. The capacity of these exchangers to fractionate material ranging from mononucleotide isomers (70) and oligonucleotides of different chain lengths (71) or composition and base sequence (72) to polynucleotides of extremely high molecular weight (73) is a striking feature of the method. Partial fractionation of ribonucleic acids can also be effected by countercurrent distribution (74), and, using this technique, Holley and co-workers (75) have obtained almost complete separation of alanine- and tyrosine-active nucleic acids in the mixture of amino acid transfer ribonucleic acids. Transfer ribonucleic acid has also been partially fractionated by chromatography on cationic starch exchanger [Smith *et al.* (76)]. Zamecnik and co-workers (77) have used preliminary chemical treatments for the same purpose. Amino acids esterifying the 3' (or 2')-hydroxyl of the terminal nucleoside of transfer nucleic acids isolated by phenol extraction were removed at pH 10. The mixture was then labelled specifically with C¹⁴-L-valine by means of the amino acid-activating enzyme. Periodate oxidation of the unesterified *cis* hydroxyl groups gave ribonucleic acids (other than the valine-protected polymers) containing a terminal dialdehyde. Addition of hydroxy-3-naphthoic acid hydrazide and coupling with tetrazotised *o*-dianisidine gave dye-bound nucleic acid and the valine specific RNA. Enrichment of the latter was obtained by fractional precipitation at a two-phase interphase; a number of other fractionation techniques could doubtless be applied, and the method (and its possible modifications) offers considerable promise. Brown (78) has used an approach of possibly greater potential value; this approach depends on the reaction of diazonium compounds with certain amino acids to form covalently linked derivatives. Preparations of transfer ribonucleic acids fully esterified with amino acids were treated with an insoluble polydiazostyrene that combined with the tyrosine- and histidine-labelled nucleic acids only. Incubation at pH 10 released the two specific polynucleotides by cleavage of the ester linkage. Further separation was then achieved by re-esterification with tyrosine by use of a purified tyrosine-activating enzyme and by repetition of the polydiazostyrene treatment. Unesterified histidine-specific nucleic acid was left in solution, while the tyrosine-specific nucleic acid was released, as before, by mild alkaline treatment of the separated polystyrene derivative. Both fractions were virtually pure with respect to their amino acid-receptor specificity. Preliminary work suggested that the valine-specific ribonucleic acid could be esterified enzymatically with the dipeptide tyrosylvaline with obvious possibilities, and this method may well be the means of obtaining a number of related specific ribonucleic acids of a size and purity that at least would permit a meaningful approach to base distribution and sequence.

The potentialities of enzymes, such as spleen and snake venom diesterases, that act in a stepwise fashion (79) have not been fully exploited

beyond demonstration of the structures of small oligonucleotides and various homopolymers of thymidylic acid [Razzell & Khorana (80)]. Since the determination of base sequence in the latter leaves little to the imagination, a more convincing demonstration of the utility of the approach would lie in the controlled liberation of mononucleotides from polymers containing several different bases, particularly since interference by differing rates of hydrolysis of different purine or pyrimidine sequences, or both, in polynucleotides of any significant length might be expected to obscure precise interpretation. Of greater reported value is the identification of incorporated labelled nucleotides as terminal or non-terminal by correlation of radioactivity with ultraviolet absorption on release of terminal nucleotides by venom diesterase (81).

The Soviet workers, particularly Belozersky and his colleagues (82 to 85), have continued their extensive investigations on the base composition of nucleic acids from a wide variety of sources (86 to 94), including higher plants, fungi, insects, and bacteria. A number of Russian publications deal with the preparative chemistry (95 to 108) of nucleic acids and nucleoproteins, and physical chemistry (109 to 112), particularly electron paramagnetic resonance (113 to 115), and the high-energy irradiation of nucleic acids and their derivatives (116 to 125) *in vitro* and *in vivo* is apparently as popular a source of research funds as elsewhere. Other aspects of the subject are dealt with in various Russian journals (126 to 134).²

NUCLEOTIDE ANHYDRIDES

Alkaline hydrolysis of adenosine-5'-triphosphate.—Lipkin and his colleagues (135) have re-examined the action of aqueous barium hydroxide on adenosine-5'-triphosphate. Many products are obtained, including adenine, adenosine, adenosine-5'-phosphate, adenosine-5'-pyrophosphate, adenosine-2'- and -3'-phosphate, adenosine-2'(or 3'),5'-diphosphates, and adenosine-3',5'-cyclic phosphate. Two types of reaction explain the formation of most of the observed products. Intermolecular nucleophilic attack by hydroxide ions or water molecules at the phosphorus atoms gives the classical type of hydrolytic products, while intramolecular reactions in which the 3'-hydroxyl group of the ribose residue attacks the phosphorus atoms give rise to the range of phosphorylated products. The presence of 2'-phosphorylated derivatives is best accounted for by hydrolysis of a 2',3'-cyclic phosphate intermediate. This would be obtained readily from the 3'-pyrophosphate formed either directly by attack of the 3'-hydroxyl at the β -phosphorus atom or possibly by phosphorylation of the 3'-hydroxyl by the γ -phosphate with subsequent nucleophilic attack of this phosphate group at the β -phosphorus of the 5'-pyrophosphate residue in an intermediate adenosine-3'-phosphate-5'-pyrophosphate. Other di- and polyvalent metal ions show catalytic

² This reviewer is greatly indebted to Dr. A. S. Spirin of the Bach Institute of Biochemistry, U.S.S.R. Academy of Sciences, Moscow, for a summary of recent work appearing in Russian journals.

effects with varying degrees of control over the nature of the reaction products; particularly noteworthy is the hydrolysis to adenosine-5'-phosphate catalysed by calcium or lanthanum ions and the almost quantitative conversion of adenosine-5'-triphosphate to adenosine-5'-pyrophosphate on stirring with a slurry of zinc oxide (136). It is likely that full elaboration of this work, now in progress, will assist considerably in the elucidation of the role or roles played by metal ions in so many of the enzymic reactions of organic phosphates.

Uridine-5'-pyrophosphate sugar peptides.—Strominger and his colleagues (137) have investigated the chemistry and biochemistry of the uridine derivatives that accumulate in penicillin-inhibited *Staphylococcus aureus* [Park & Johnson (138)]. The pentapeptide derivative has been identified as L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine linked from the amino group of the L-alanyl residue to the carboxyl group of the lactic acid residue in uridine diphosphate N-acetylmuramic acid. Biochemical confirmation of the structure has been obtained by a study of the biosynthesis of the compound by use of metabolic blocks at specific points, which results in accumulation of the precursors of the pentapeptide derivatives. Single amino acids are added to the lactic acid derivative of uridine diphosphate N-acetylglucosamine in a stepwise fashion up to the tripeptide derivative, which is then converted directly to the pentapeptide by the addition of the dipeptide D-alanyl-D-alanine (139 to 143).

Other derivatives.—Cytidine-5'-phosphate N-acetylneuraminic acid has been isolated from *Escherichia coli* (144), and, while not an anhydride strictly speaking, it is a member of what may be a new class of activated nucleotide derivatives.

A number of guanosine-5'-pyrophosphate derivatives have been isolated and identified. These include guanosine diphosphate-mannose (145), -fucose (146), -glucose (147), -fructose (147), and -colitose (3,6-dideoxy-L-galactose) (148).

In addition to the ribonucleoside-5'-pyrophosphate-X derivatives that have been known for some time, several analogous deoxynucleotide anhydrides have been observed. Deoxycytidine diphosphate choline has been isolated from sea urchin eggs (149), and both this nucleotide and the ethanolamine derivative are present in extracts of calf thymus (150). Several thymidine diphosphate-sugar compounds (151), such as thymidine diphosphate-rhamnose (152, 153) and thymidine diphosphate-mannose (153), have been isolated from various bacteria.

Synthesis of nucleotide anhydrides.—Methods for the synthesis of nucleotide coenzymes and other anhydrides have developed rapidly since the early classical approaches. Carbodiimides, particularly dicyclohexylcarbodiimide, have proved useful, although they are subject to a number of drawbacks, such as the lack of reproducibility of reported yields in certain cases (154) and the formation of all three possible anhydrides from a given pair of acids when the anions are comparable nucleophiles. The method is also

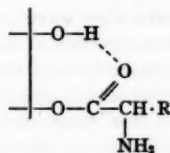
of limited value for the synthesis of coenzymes such as flavin-adenine dinucleotide, uridine diphosphate-glucose, and coenzyme A because of cyclisation and other undesirable reactions. By the use of nucleoside-5'-phosphoramidates, however, relatively efficient syntheses of a number of coenzymes of this type have been developed by Khorana and co-workers [Moffatt & Khorana (155)] and by Todd and co-workers [Clark *et al.* (156)]. More recently, Michelson (157) has developed rapid simple and effective methods that involve controlled nucleophilic displacement of one component of a nucleotide anhydride by a third acid (an anion-exchange procedure analogous to the enzymic synthesis of nucleotide coenzymes) which is of wide applicability.

Phosphoramidates.—When protonation of a phosphoramidate occurs at the nitrogen, rather than an oxygen atom, the electrophilic character of the phosphorus atom is greatly increased, and such phosphoramidates can be effective phosphorylating agents, though reactivity is low and, in general, they are susceptible to attack only by such strong nucleophiles as anions. Thus, the reaction of benzyl hydrogen phosphoramidate with pyridinium adenosine-5'-phosphate yielded the benzyl ester of adenosine-5'-pyrophosphate. Further reaction of adenosine-5'-pyrophosphate with benzyl hydrogen N-cyclohexyl phosphoramidate gave the monobenzyl ester of adenosine-5'-triphosphate (156). In an alternate approach, Chambers & Moffatt (158) developed syntheses of nucleoside-5'-phosphoramidates by the action of dicyclohexylcarbodiimide on a mixture of the nucleotide and primary or secondary base. In addition to the synthesis of nucleoside-5'-pyrophosphates (159, 160) and -triphosphates (161), these intermediates proved useful for the synthesis of diesterified pyrophosphates, such as uridine diphosphate-glucose (155), uridine diphosphate-glucuronic acid, and guanosine diphosphate-mannose (162), from the appropriate nucleoside phosphoramidate and sugar phosphate. In the same way, reaction of dicyclohexylguanidinium adenosine-5'-phosphoramidate with riboflavin-5'-phosphate gave flavin-adenine dinucleotide (155). Similarly, treatment of pantethine-4-phosphate with adenosine-5'-phosphoromorpholidate gave dephosphocoenzyme A, while the same procedure applied to adenosine-2',3'-cyclic phosphate-5'-phosphoromorpholidate gave a product that on treatment with dilute acid to open the 2',3'-cyclic phosphate yielded a mixture of coenzyme A and isoenzyme A (163). In a minor modification of the phosphoramidate approach, imidazolium adenosine-5'-imidazol-1-ylphosphonate prepared from adenosine-5'-phosphate and 1,1'-carbonyl di-imidazole has been employed (164).

Anion exchange.—Khorana & Vizsolyi (165) have described the formation of P^1, P^2 -dinucleoside-5'-pyrophosphates on treatment of the nucleotide with acetic anhydride; this approach is analogous to the earlier work using trifluoroacetic anhydride (166). With excess reagent, adenylyl acetate is obtained; but under anhydrous conditions with limited amounts of acetic anhydride the dinucleoside-5'-pyrophosphate is slowly formed. Thermodynamic stability and kinetic preference have been advanced by Khorana &

Vizsolyi (165) as explanations for the formation of the pyrophosphate derivative and its resistance to nucleophilic attack by acetate ion. More pertinent explanations of their results may be derived from the concepts outlined by Todd (66). Thus, nucleophilic displacement of the stronger acetate anion ($pK \sim 4.8$) from an intermediate adenylyl acetate by adenosine-5'-phosphate ($pK \sim 6$) may be expected. Since the additive property of the carbonyl group is an activating effect, anion exchange by nucleophilic attack at the nucleotide phosphorus atom is considerably slower than exchange by attack at the carboxyl component. Under anhydrous conditions, however, treatment of adenylyl acetate with adenosine-5'-phosphate results in the formation of the dinucleoside pyrophosphate; the more rapid exchange with acetate merely produces adenylyl acetate. Resistance of diadenosine-5'-pyrophosphate to nucleophilic attack by acetate ion may be expected also, since cleavage would involve liberation of a less stable anion. However, degradation of the pyrophosphate by acetic anhydride [as well as by trifluoroacetic anhydride (167, 168)] will occur by the nucleophilic attack of the acetate ion on an intermediate acetyl pyrophosphate. It may be noted that it is the additive character of the carbonyl group that results in mixed anhydrides such as adenylyl acetate acting as acylating agents, not only when the phosphate moiety is a stronger acid than the carboxylic acid (in which case displacement of the more stable anion occurs), but also when the carboxylic acid is stronger than the phosphoryl dissociation liberated under the pH conditions employed. Again, treatment of thymidylyl acetate with phosphoric acid in pyridine solution gives thymidine-5'-phosphate (rather than the pyrophosphate) and acetyl phosphate (165). Further attack by inorganic phosphate then occurs at both the carboxyl and phosphate groups of acetyl phosphate, but only in the latter case is a new product, inorganic pyrophosphate, formed. Amino acid anhydrides of nucleotides behave in a similar fashion though, being anhydrides of stronger acids, they are considerably less stable than the corresponding anhydrides with carboxylic acids unless the amino function is blocked. This increase in reactivity is also evident in the 2' (or 3')-amino-acyl esters of ribonucleotides (and polynucleotides) compared with the 2' (or 3')-acyl derivatives, but even these are considerably less stable (169) than simple alkyl esters that lack an adjacent hydroxyl group, such as the 3'- or 5'-esters of the deoxyribonucleosides. A possible explanation may involve hydrogen bonding of the vicinal *cis* hydroxyl group with the carbonyl group, thereby facilitating nucleophilic attack at this carbon (III).³

³ Infrared spectra of 2',3'-di-O-acetyl adenosine and 3',5'-di-O-acetyl adenosine indicate hydrogen bonding in the latter compound (171). However, evaluation of kinetic data obtained from studies of an extensive series of model compounds by Zachau & Karau (225, 226) and Wieland and co-workers (227) shows that other factors are involved. The reactivity of the ester linkage is strongly influenced by the acid component and by the ring oxygen of the sugar as well as by the neighbouring hydroxyl group. Participation of hydrogen bonds and inductive and steric effects are discussed in Zachau & Karau (226).

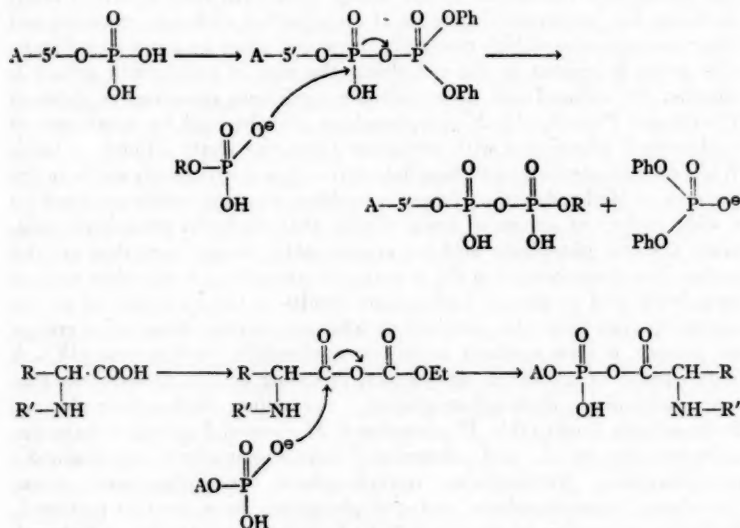


III

The theoretical basis of the reactions of nucleotide anhydrides of various kinds has been applied with considerable success to the synthesis of nucleotide coenzymes [Michelson (157)]. Early work with fully esterified intermediates was unsuccessful because of uncontrolled exchange reactions and other consequences of high reactivity. However, when an unprotected ionisable group is present in the anhydride, the ease of nucleophilic attack is considerably reduced and, under suitable conditions, quantitative yields of P^1 -diphenyl- P^2 -nucleoside-5'-pyrophosphate are obtained by treatment of nucleoside-5'-phosphates with tetraphenyl pyrophosphate [Michelson (63)]. While such triesterified pyrophosphate derivatives are relatively stable in the presence of hindered tertiary bases, in pyridine they are rapidly attacked by a wide variety of anions of acids weaker than diphenyl phosphoric acid. Since diphenyl phosphoric acid is a considerably stronger acid than are the nucleotides, displacement of the more stable anion by a nucleophile such as phosphoric acid or glucose-1-phosphate results in the formation of a new nucleotide anhydride; this, particularly when two or more dissociating groups are present, is then resistant to further nucleophilic displacement (IV). A large number of nucleotide anhydrides, including uridine diphosphate-glucose, adenosine diphosphate-glucose, thymidine diphosphate-glucose, flavin-adenine dinucleotide, P^1 -adenosine-5'- P^1 -glycerol-2-pyrophosphate, dephosphocoenzyme A, and adenosine-5'-sulphatophosphate, adenosine-5'-pyrophosphate, -triphosphate, -tetraphosphate, -pentaphosphate, -hexaphosphate, -heptaphosphate, and -polyphosphate, have been so prepared. Similarly, conversion of adenosine-2'(3'),5'-diphosphate to the P^1 -diphenyl P^2 -adenosine-(2',3'-cyclic phosphate)-5'-pyrophosphate followed by reaction with pantethine-4-phosphate gave a 2',3'-cyclic phosphate intermediate from which coenzyme A was readily obtained. Nucleoside-5'-phosphoramidate derivatives were obtained quantitatively on treatment of the intermediate diphenyl phosphate anhydride with primary or secondary amines, and, analogous to the anion exchange reactions, preferential nucleophilic attack at the nucleotide phosphorus occurred with liberation of the stronger anion. In general, the intermediate anhydride may contain any esterified phosphoric acid that is stronger than both the nucleotide and the attacking anion. Sulphonyl anhydrides, however, do not appear to be satisfactory because of the rapid conversion of the nucleotide to dinucleoside-5'-pyrophosphate, even in the absence of base catalysis. Letters & Michelson (170), in a further variation of this general approach, treated carbobenzyloxymino acids with ethyl chloroformate to give the ethyl carbonate anhydride. Addi-

tion of nucleoside-5'-phosphate then gave aminoacyl nucleotides in high yield. The stronger additive properties of the aminoacyl carbonyl group ensure specific formation of the aminoacyl nucleotide rather than the relatively stable ethyl carbonate-nucleotide anhydride. The methods have also been applied to the synthesis of oligonucleotides terminating in a 5'-pyrophosphate group and oligonucleotide-peptide anhydrides.

Similar anion exchange mechanisms may be postulated for the slow conversion of acid salts of nucleoside (and deoxynucleoside)-5'-pyrophosphates in the solid state to mixtures of nucleoside-5'-triphosphate and nucleoside-5'-phosphate [Michelson (171)].



IV

Oxidative phosphorylation.—In a study of model systems of oxidative phosphorylation, Clark, Hutchinson & Todd (172) have successfully prepared adenosine-5'-pyrophosphate by bromine oxidation of a mixture of adenosine-5'-phosphate and 2,3-dimethylnaphtha-1,4-quinol monophosphate or of inorganic phosphate and the naphtha-1,4-quinol ester of adenosine-5'-phosphate.

POLYNUCLEOTIDE SYNTHESIS

The first chemical synthesis of a compound containing a true internucleotide linkage [Michelson & Todd (173)] used the classical approach of base-catalysed reaction between a phosphorochloridate and suitably protected

alcohol. Treatment of 3'-O-acetyl thymidine with 5'-O-acetyl thymidine-3'-benzylphosphorochloridate gave the fully protected dinucleoside phosphate from which protecting groups were removed to yield thymidylyl-3':5'-thymidine. An analogous procedure using thymidine-3'-benzyl phosphorochloridate-5'-dibenzyl phosphate gave the dinucleotide thymidylyl-5':3'-thymidine-5'-phosphate. Extension to the ribose series resulted in the synthesis of adenylyl-2':5'-uridine (169). While this work demonstrated the feasibility of oligonucleotide synthesis, it was clear that simpler and more effective methods would be necessary. An early observation by Christie and co-workers (166) indicated that treatment of uridine-5'-phosphate with excess dicyclohexylcarbodiimide gave rise to materials that were probably of higher molecular weight than the expected dinucleoside pyrophosphate. Further work by Khorana and his group [Tener *et al.* (71)] showed that the reaction of thymidine-5'-phosphate with either toluene-*p*-sulphonyl chloride or, preferably, dicyclohexylcarbodiimide in anhydrous pyridine yielded a number of polymeric products. Fractionation of the extremely complex mixture showed the presence of two major series of compounds which were identified as linear oligodeoxynucleotides with 3',5'-phosphate diester linkages and a related series of cyclic oligonucleotides derived from the linear compounds by phosphorylation of the terminal 3'-hydroxyl by the 5'-phosphate group at the other end. Chromatographic methods suggested the presence of polymers that contain up to 11 nucleotide units, and oligonucleotides up to the pentanucleotide were isolated in a relatively pure condition. In like manner, the polymerisation of thymidine-3'-phosphate (after conversion to P¹,P²-dithymidine-3'-pyrophosphate) by treatment with excess dicyclohexylcarbodiimide gave a mixture of products. Even lower yields of the linear thymidine oligonucleotides bearing 3'-phosphate end groups were obtained, together with the homologous series of cyclic oligonucleotides (64). Two other series of nucleotide components were also observed, one of which consisted of polymers containing P¹,P²-dithymidine-3'-pyrophosphate linkages in addition to the normal 3',5'-internucleotide linkage. Members of the other series of homologous products were tentatively identified as oligonucleotides that contain a 5'-deoxy-5'-quaternary pyridinium derivative of the terminal nucleoside. While the mechanisms of the reactions involved have not been fully elucidated as yet, Todd (66) has suggested that monomeric metaphosphate derivatives are the active intermediates. Further reaction of nucleoside-5'-monomeric metaphosphate with pyridine to give active phosphoramidates, or conversion to tri- and higher metaphosphates, might also be expected (V).

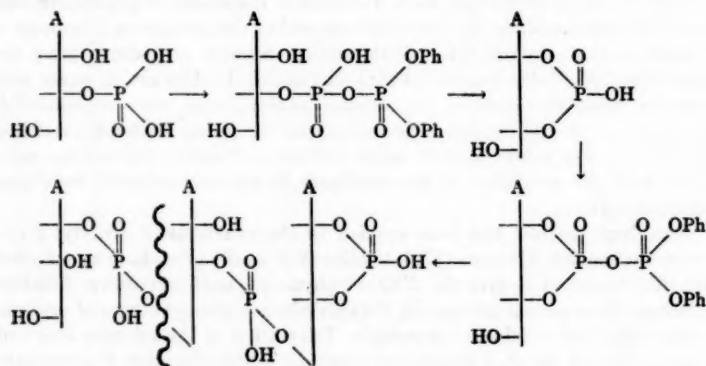
In spite of the complexity of the product mixture, the procedure has been claimed as a new and general method for the synthesis of the 3',5'-internucleotide linkage in which the phosphomonoester grouping is activated directly (71). Considerable naïveté is required for unqualified acceptance of this claim. Greater success was achieved in the stepwise synthesis of small oligodeoxynucleotides by use of suitably protected nucleotide and nucleoside

derivatives (174). The reaction of 3'-O-acetylthymidine-5'-phosphate and 5'-O-tritylthymidine with dicyclohexylcarbodiimide followed by removal of protecting groups yielded a mixture containing thymidylyl-5':3'-thymidine, thymidine-5'-phosphate, thymidine, and P^1, P^2 -dithymidine-5'-pyrophosphate from which the required product was separated by paper chromatography. Deoxycytidylyl-5':3'-thymidine and deoxyadenylyl-5':3'-thymidine were similarly prepared, whereas replacement of 5'-O-acetylthymidine by thymidine-5'-dibenzyl phosphate yielded the dinucleotide deoxyadenylyl-5':3'-thymidine-5'-phosphate. Alternative procedures in which a protected dinucleoside phosphate is phosphorylated at the sole free sugar hydroxyl group have been developed (174). General application to the synthesis of oligodeoxynucleotides would appear to be markedly restricted because of the high reactivity of the metaphosphate intermediates and interference by functional groups of certain purines and pyrimidines [Gilham & Khorana (175)]. Nevertheless, the approach has been extended to the synthesis of two trideoxynucleoside diphosphates, which were isolated in relatively pure condition by paper chromatographic methods. Thus, treatment of thymidylyl-5':3'-(5'-O-trityl)-thymidine with 3'-O-acetyl thymidine-5'-phosphate and dicyclohexylcarbodiimide followed by removal of the protecting groups gave thymidylyl-5':3'-thymidylyl-5':3'-thymidine. Similar procedures gave deoxycytidylyl-5':3'-deoxyadenylyl-5':3'-thymidine. In this case, however, side reactions, possibly involving the adenine amino group, were encountered, and removal of the triphenylmethyl-protecting group without extensive degradation was achieved with some difficulty. Possible limitations associated with the solubility of intermediates in suitable solvents were also indicated (175).

The same method has been applied to the synthesis of uridylyl-5':3'-uridine by Smith & Khorana (176). Uridine-3',5'-cyclic phosphate was treated with dihydropyran to give the 2'-O-tetrahydropyranyl derivative. Alkaline hydrolysis then gave a mixture of 2'-O-tetrahydropyranyl ethers of uridine-3'-phosphate and uridine-5'-phosphate. Tritylation of the mixture followed by separation of the 2'-O-tetrahydropyranyl-5'-O-trityluridine-3'-phosphate from the unchanged 5'-phosphate derivative and reaction with 2',3'-di-O-acetyluridine and dicyclohexylcarbodiimide and then removal of protecting groups gave the diuridine phosphate containing a 3',5'-internucleotide linkage as in the naturally occurring ribonucleic acids.

When the nucleotide contains an unprotected *cis* hydroxyl group adjacent to the phosphate residue, the metaphosphate approach cannot be employed because of the ready formation of cyclic phosphates that, being phosphate diesters, can no longer be converted to metaphosphate intermediates. However, the ribonucleotides can be polymerised readily and quantitatively by means of a modified approach with fully esterified mixed anhydrides, as developed by Michelson (177). The polymerisation process involves the initial formation of a P^1 -nucleoside-2'(3')- P^2 -diphenyl pyrophosphate by the action of diphenyl phosphorochloridate on the readily

available ribonucleoside-2'- or -3'-phosphates. Intramolecular phosphorylation by the weaker of the two acids that form the anhydride occurs rapidly to give the 2',3'-cyclic phosphate that, with more reagent, then gives a second transient unsymmetrical anhydride, P¹-nucleoside-2',3'-cyclic-P²-diphenyl pyrophosphate; the latter rapidly polymerises by intermolecular phosphorylation of the free hydroxyl group with liberation of diphenyl phosphate. Final cleavage of the polymeric phosphate triester to give the diester, which occurs spontaneously in aqueous solution, can result in the formation of 2',5'- or 3',5'-internucleotide linkages or degradation. In practice, degradative cleavage is slight. Using this approach, homopolymers of all the major ribonucleotides (including 5-ribosyluracil phosphate) have been prepared with chain lengths of up to 10 to 15 nucleotides (178, 179). Because of the nature of the process, these polymers do not contain 3',5' internucleotide linkages exclusively, some 50 per cent being the isomeric 2',5'. Nevertheless, the synthetic guanylic oligonucleotides, particularly those of chain length five to seven nucleotides, proved remarkably effective for the induction of Streptolysin-S formation in streptococci (180) (VI).



VI

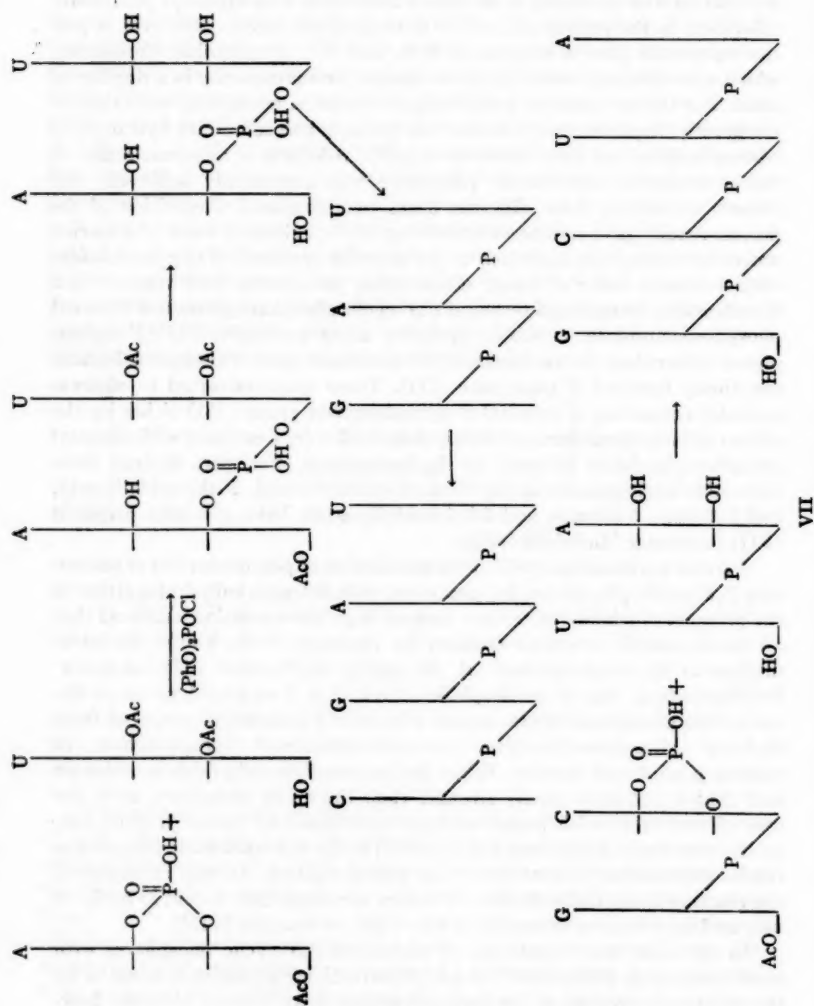
A number of copolymers were also prepared. Copolymerisation of adenylic and uridylic acids gave the mixed polynucleotides with presumably a random distribution of adenine and uracil, whereas partial polymerisation of the two nucleotides separately to oligonucleotide material of average chain length three nucleotides followed by copolymerisation of the mixture of oligonucleotides gave polymers with traces of purines and pyrimidines (178). A third form of copolymer was obtained by polymerisation of the dinucleotide adenylyl-3':5'-uridine-3'-phosphate to give material with a defined arrangement of alternate adenylic and uridylic acids (179).

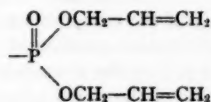
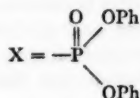
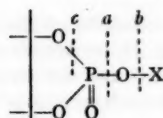
The same general procedure has been applied to the stepwise synthesis of a large number of oligonucleotides containing several different bases in a

defined order. Treatment of the nucleoside-2'(3')-phosphate with ethyl chloroformate in aqueous solution gave the cyclic phosphate that after acetylation was converted to the mixed anhydride with diphenyl phosphorochloridate in the presence of a 2',3'-di-O-acetylnucleoside. Removal of protecting groups gave a mixture of 2',5'- and 3',5'-dinucleoside phosphates, which were then separated by ion exchange chromatography in a number of cases. For the synthesis of higher oligonucleotides, advantage was taken of the greatly disparate reactivities of the primary and secondary hydroxyls of ribonucleosides and their derivatives (179). Addition of mononucleotide to the unprotected dinucleoside phosphate was successfully achieved, and trimers containing three different bases were prepared. Repetition of the process readily gave tetramers containing the four classical bases in a specific order. In an alternate approach to the stepwise synthesis of oligonucleotides with a defined order of bases, dinucleoside phosphates were treated with dinucleotides containing a terminal 2',3'-cyclic phosphate group and diphenyl phosphorochloridate. A simple variation using nucleoside-2'(3'),5'-diphosphates rather than the nucleoside-2'(3')-phosphate gave true oligonucleotides containing terminal 5'-phosphate (171). These were converted to oligonucleotides containing a terminal 5'-pyrophosphate group (181) either by the anion exchange procedure previously described or by treatment with dibenzyl phosphorochloridate followed by hydrogenolysis. Polymers derived from nucleotide analogues, including N¹-methyluridylic acid, 5'-thiouridylic acid, and 5-chloro-, 5-bromo-, and 5-iodouridylic acids, have also been prepared (VII) [Letters & Michelson (182)].

Further examination (182) of the mechanism of polymerisation of nucleoside-2',3'-cyclic phosphates by treatment with different anhydrides either in the presence of a hindered tertiary base or in pyridine solution indicated that all three possible reactions denoted by cleavage of the anhydride intermediate at the bonds marked (a), (b), and (c) in Formula VIII can occur. Polymerisation, that is, nucleophilic attack of a 5'-hydroxyl group at the nucleotide phosphorus atom, occurs with mixed anhydrides prepared from diphenyl phosphorochloridate, tetra-*p*-bromophenyl pyrophosphate or toluene-*p*-sulphonyl chloride. When the intermediate anhydride contains an acid that is not significantly stronger than the cyclic phosphate, as in the case of treatment with tetrabenzyl pyrophosphate or tetraallyl pyrophosphate, then nucleophilic attack of hydroxyl at the non-nucleotide phosphorus results in phosphorylation rather than polymerisation. As may be expected, the reaction is markedly slower and occurs to a significant extent in pyridine only and may involve formation of a pyridinium complex (VIII).

In the same way, treatment of nucleoside-2',3'-cyclic phosphates with acetic anhydride yields the 5'-O-acetyl derivatives; acylation is assisted by the additive properties of the carbonyl group. With benzoyl chloride, however, the third mechanism may occur, that is, nucleophilic attack of hydroxyl at the phosphorus atom and cleavage of bond (c) to give an intermediate oligo(nucleotide-benzoyl anhydride) which is then hydrolysed to oligonucleo-





VIII

tide, since considerable polymerisation was obtained in the presence of hindered tertiary base. The pyridine-catalysed reaction, however, effected more benzylation than polymerisation. An even stronger solvent directive effect, but with reverse results, was observed with a group of anhydride reagents typified by methane sulphonyl chloride. In dioxan in the presence of hindered tertiary base, only 5'-O-sulphonyluridine-2',3'-cyclic phosphate was obtained, whereas extensive polymerisation occurred in pyridine.

The nature of the products from polyesterified nucleotide anhydrides of the type described is thus a function of the components of the anhydride and, in particular, of the non-nucleotide moiety (though some variation in the behaviour of different nucleotides was observed), of the nature of the solvent (the dielectric properties of the environment may possibly affect the electronic distribution in the anhydride), and of the presence or absence of base catalysis. In some respects, the chemical control and direction of the course of these reactions is analogous to the enzymic control that is evident in a number of biochemical reactions involving anhydrides of nucleoside-5'-phosphates. Hydrogen bonding or metal chelation, or both, of substrate to enzyme presumably has a considerable directive effect on nucleophilic attack at a particular phosphorus atom. Further analogies are available in the chemical synthesis of nucleotide coenzymes by controlled nucleophilic anion exchange.

PHYSICAL PROPERTIES OF OLIGONUCLEOTIDES

Ultraviolet absorption.—Examination of the chemically synthesised oligoribonucleotides and small polynucleotides showed that they possess many of the physical properties formerly assumed to be associated solely with relatively high molecular weight polynucleotides. One of the more

striking of these properties is the effect of polymerisation on the ultraviolet absorption of the purine and pyrimidine bases. Hypochromic effects are observed in a wide range of oligonucleotides (183) and can be quite large even in dinucleotides [Michelson (184)]. In a given homologous series, the effect reaches a limit at chain lengths of five to six nucleotides and is a function not only of the composition of the entire molecule but also the order of bases. Considerable variation of hypochromicity with pH occurs, and again the effect of pH on the ultraviolet absorption of oligonucleotides relative to the absorption of the component mononucleotides at the same pH is a composite of many different curves characteristic of both the composition and distribution of bases (184). In particular, the hypochromicity of dinucleotides that contain adjacent aminopurines or aminopyrimidines is considerably reduced in acidic solution, whereas in polymers that contain adjacent 6-keto groups an increase in absorption occurs at alkaline pHs when the heterocyclic base is fully ionised. Hypochromicity is not restricted to 3',5'-internucleotide-linked polymers but is also shown by 5',5'-phosphodiester and 2',5'-linked isomers. Quite marked hypochromic effects (15 to 40 per cent) are shown by ω -di-9-purinyl ethanes, particularly, hexanes (185), and dinucleoside-5'-pyrophosphate (171). In general, 2',5'-isomers show greater hypochromic effects than the 3',5'-derivatives. Different ϵ values are shown by isomeric dinucleoside phosphates such as adenylyl-2':5'-uridine and uridylyl-2':5'-adenosine; though less pronounced, such differences are also evident in 3',5'-linked oligonucleotides of identical base composition but of different base sequence [Michelson (186)]. Rather large effects, larger in fact than those shown by adenine derivatives, are shown by dinucleoside phosphates derived from 6-dimethylaminopurine at all pHs (181), and it is unlikely that hydrogen bonding plays a significant role in the hypochromicity of oligonucleotides, particularly since the effect is not sensitive to increases in temperature [Michelson (171)]. Moreover, the disappearance of hypochromicity in dinucleotides such as adenylyl-3':5'-uridine and adenylyl-3':5'-cytidine-3'-phosphate on saturation of the 4,5 double bond in the pyrimidine by hydrogenation or photolysis suggests that the π electron systems of the purine and pyrimidine bases are essential for manifestation of the effect [Janion & Shugar (187); Wierzchowski & Shugar (188)]. In the case of the photolysed derivatives, thermal reversal leads to a complete recovery of hypochromicity. Similar results were obtained with guanylyl-uridylyl adenosine (GpUpA), but with the isomeric uridylylguanylyl adenosine (UpGpA) the dihydro or photolysed product still exhibited a lowered absorptivity resulting from the adjacent purine residues (180).

The hypochromicity of a given oligonucleotide is thus a measure of the interaction between the bases; the interaction is dependent on the base overlap and interplanar distances. As may be expected, these factors are not identical in isomeric dinucleoside phosphates, since the direction of the linkage controls the displacement necessary for closest approach of the purine and pyrimidine bases.

While other explanations have been offered, the most plausible interpretation is that even in oligonucleotides the purine or pyrimidine bases, or both, are stacked above each other and that direct interaction between adjacent ring systems not only stabilises this configuration but also has a profound effect on the ultraviolet absorption that is characteristic of the entire molecule rather than a simple summation of the independent absorptivities of the component mononucleotides (184). Even in dinucleotides and dinucleoside phosphates, this interaction results in a restricted rotation about the internucleotide linkage. In addition to the stacking of bases in polynucleotides, the interplanar interaction may also result in a helical twist along the sugar phosphate axis, assuming closest approach and maximum overlap of the π electron systems. This could give considerable stability to double helical structures in the absence of interstrand hydrogen bonds [cf. the reversible titration of deoxynucleic acid at or below 0° (189, 190)]. A possible cause of the effect of ionisation of the purine and pyrimidine bases on hypochromicity may be the increased lateral displacement or interplanar separation caused by repulsion between similarly charged purines or pyrimidines. Since two opposing effects are present, that is, strain set up by the charged phosphate groups and interaction of the bases (186), the position of this balance in terms of the precise stereochemical structure of the molecule will undoubtedly be greatly influenced by the degree of ionisation of the phosphate residues (affected by pH, ionic strength, and nature of the cations present) as well as the state of the bases. In general, any process that increases the interaction of the purine and pyrimidine rings, such as contraction of the macromolecule or metal chelation between the ring systems, should increase the hypochromic effect, whereas a further diminution in absorption may be expected as a result of hydrogen bonding within the same strand or between different polynucleotide chains. Tinoco (191) has presented a theoretical treatment of the hypochromic effect in polynucleotides.

Some support for the concept that defined conformations begin to appear even in oligonucleotides is provided by a comparison of the electrophoretic mobilities of isomeric 2',5'- and 3',5'-dinucleoside phosphates (179). The results suggest that the former are more compact than the latter because of stronger interaction between the bases.

Interaction of oligonucleotides with planar basic dyes.—The aggregation of certain basic dyes that fail to obey Beer's Law in aqueous solution has long been held to occur by the stacking of the planar aromatic molecules, the micelle being held together by London dispersion forces between the π electron systems with consequent change in the visible absorption spectra. Binding of such dyes by nucleic acids causes similar effects in dilute solution; the phosphoryl groups of the polynucleotide chains are generally regarded as the major binding sites. The synthetic polymers (of average chain length approximately six nucleotides) were likewise effective in producing pronounced changes in the absorption spectra of rosaniline, toluidine blue, and acridine orange when equimolar amounts of dye and polymer phosphate at

low ionic strength were used (178, 179). From a study of the size of the effect shown by different oligonucleotide fractions, it would seem that significant interaction occurs at chain lengths of eight to ten nucleotides, though, because of the mixed nature of the 2',5'- and 3',5'-internucleotide linkages, the arrangement of the binding sites is not as uniform as in the natural polynucleotides and, in a given series of homologues containing 3',5'-linkages exclusively, interaction may well occur at even lower chain lengths. The nature of the purine or pyrimidine base in the polymer chain has a profound effect on the induced metachromasy of the dye. Whether this is an indication of secondary stereospecific binding sites or a reflection of the extent of ordered configuration in the various homopolymers is not yet known. The effectiveness of homopolymers of comparable chain lengths is in the ascending order polyuridylic < polycytidylic < polyadenylic < polyguanylic acids, an order which is also observed for polynucleotide-induced quenching [Steiner & Beers (192)] of the fluorescence of acridine orange. Polyglycerophosphate was quite without effect on the dyes under the general conditions used, but marked interactions of acridine orange and α -poly-L-glutamic acid, comparable with the metachromasy induced by polyguanylic acid, was observed. Similar interaction of a wide variety of basic dyes with apurinic acid, which contains relatively short stretches (up to ten nucleotides) of poly-pyrimidine deoxynucleotides, has been studied by Adamiec & Shugar (193).

Pour-El & Dekker (194) have described the interaction of mercuric chloride with oligonucleotides. In this case, the heterocyclic bases are probably directly involved. While negligible changes in the ultraviolet absorption spectra occurred on addition of mercuric chloride to adenylyl-3':5'-adenosine, the synthetic polyadenylic acids showed an increasing degree of interaction with increase in chain length, as evidenced by the bathochromic shift, and decrease in absorptivity.

Apparent pK values.—No significant changes in apparent pK were observed with polyuridylic acids or polycytidylic acids (of short chain lengths), but spectrophotometric titrations of polyguanylic acids showed marked shifts of the apparent pK values of the amino and keto groups to higher values (178). A similar shift of the guanine —NH—CO— dissociation was observed on polymerisation of the dinucleotide guanylyl-3':5'-cytidine-3'-phosphate (G3'P5'C3'P). While differences might be expected as a result of charge effects and, indeed, with respect to the cytosine amino dissociation, they have been observed between cytidine-2'-phosphate and cytidine-3'-phosphate (195), between isomeric 2'-5' and 3'-5' dinucleoside phosphates (179), and between dinucleoside phosphates and dinucleotides (179), such differences are small and to judge from the oligocytidylic acids polymerisation per se has a negligible effect. The possibility therefore arises that such shifts (previously observed in deoxynucleic acids) are primarily the result of hydrogen bonding either within the strand or between distinct oligonucleotide chains.

Other properties.—Interaction between the chemically synthesised polyadenylic and polyuridylic acids was not observed, but partial interaction between polycytidylic and polyguanylic acids was indicated by counter-current distribution and paper electrophoresis of the homopolymers and their mixture (179). This interaction, accompanied by a small decrease in ultraviolet absorption indicative of hydrogen bonding, is presumably similar to that occurring with the enzymically synthesised polymers of high molecular weight [Warner (196)] and is a further demonstration of restricted rotation about the internucleotide linkage in oligonucleotides. Again, because of the mixed 2',5'- and 3',5'-internucleotide linkages, complete interaction even for material of longer chain length would not be expected. Subsequently, hydrogen bonding between high molecular weight polyuridylic acid and oligoadenylic acids of chain lengths two to ten nucleotides (containing the natural 3',5'-phosphodiester linkage exclusively) was demonstrated (197). The stability of the complexes increased markedly with the oligonucleotide chain lengths, as indicated by dissociation temperatures determined by changes in ultraviolet absorption and optical rotation, and a maximum decrease in absorptivity was obtained at base ratios of uracil:adenine = 2:1, suggesting the formation of a complex similar to the triple-stranded helical complex containing two polyuridylic acid chains and one polyadenylic acid. Similar interaction was also observed with high molecular weight polyadenylic acids and oligouridylic acids (198).

Further evidence of interaction between adjacent bases in oligonucleotides is suggested by the increased sensitivity of thymine oligonucleotides and oligouridylic acids to ultraviolet irradiation relative to the mononucleotides [Wierzchowski & Shugar (188, 199)]. It may be noted too that an increased sensitivity is shown by apurinic acid compared with undenatured deoxyribonucleic acid (200). The results of the photochemical work (188) provided confirmation of the hypothesis that biological photoreactivation proceeds via reversible photolysis of pyrimidine rings in polynucleotide chains.

Shugar & Baranowska (201) have described an interesting effect of ultraviolet irradiation on films of polyuridylic acids (average chain length approximately six nucleotides), deoxyribonucleic acid, and apurinic acids. Water-insoluble fibres are formed which presumably result from photochemically-induced 4(5)-4(5) cross-linking between individual oligonucleotide chains leading to multistrand polymer lattices. Such cross-linking occurs on ultraviolet irradiation of frozen solutions of thymine to give a dimer [Beukers & Berends (202)].

The general properties of oligonucleotides suggest that secondary structural characteristics are not confined to material of high molecular weight and that at chain lengths of seven to ten nucleotides specifically favoured conformations may begin to appear. A second level of organisation occurs when virtually complete resistance of movement of the bases (relative to that possible in oligonucleotides) is effected by the formation of hydrogen-

bonded structures. A lower limit of chain length for the formation of helical structures by interaction between short strands is probably some 10 to 20 nucleotides.

PHYSICAL CHEMISTRY OF POLYNUCLEOTIDES

A critical account of the physical chemistry of nucleic acids must be left for another year. The formation of double and triple helical structures from enzymically synthesised polyribonucleotides has been reviewed by Rich (203). Much larger hypochromic effects are shown in general by the high molecular weight polymers compared with analogous oligonucleotide materials, and, since the effect is temperature sensitive to a large extent, it is likely that considerable hydrogen bonding exists in the single-stranded structures [Warner & Breslow (204)]. Details of the x-ray diffraction studies of lithium deoxynucleic acid by Langridge and his colleagues (205, 206) have appeared, while Doty and co-workers [Marmur & Doty (207); Sueoka *et al.* (208)] have shown that the density and configurational stability of deoxyribonucleic acid are functions of the guanine-cytosine content. Shear degradation of deoxyribonucleic acid under conditions commonly used for isolation can be significant and leads to material of uniform molecular weight (209 to 211). Cavalieri and co-workers (212) have questioned the nature of the subunit of deoxyribonucleic acid from *E. coli*. In a study of the nature and quantitative determination of the hyperchromic effect, Spirin, Gavrilova & Belozersky (213) conclude that the ultraviolet absorption of deoxyribonucleic acid is affected by at least three structural factors: direct interplanar interaction, hydrogen bonding between complementary chains, and random hydrogen bonds between parts of the same complex or different chains.

Secondary structure in ribonucleic acids is now receiving some attention, and a number of groups consider that many ribonucleic acids contain some organised helical regions (214 to 217) involving hydrogen bonding, as shown by thermally induced hyperchromic effects and optical rotation changes. Biochemical methods (action of polynucleotide phosphorylase) also indicate that the major part of preparations of "soluble" ribonucleic acid from rabbit liver has an enzyme-resistant conformation (218). In a series of papers (219 to 222), Spirin, Belozersky, and co-workers have examined high molecular weight ribonucleic acid by a number of physical methods. Their results also indicated that regions of organised helical structure exist as a result of intra-strand hydrogen bonds. In addition to the secondary structural characteristics, tertiary structure involving folding and coiling of the whole chain of tobacco mosaic virus ribonucleic acid into a compact rod is discussed. The organisation of nucleic acids within nucleoproteins of various kinds has been examined in a study of the hypochromic effect (with respect to the nucleic acid) in the nucleoprotein compared with that of the free polynucleotide (223, 224).

The main chemical problems awaiting full solution include the fractiona-

tion of nucleic acids into components that can be regarded as homogeneous molecular species, the determination of base sequence in such polynucleotides by methods considerably more refined than those now available, and the development of superior polymerisation procedures for the synthesis of specific high molecular weight polynucleotides. Despite the pessimism of some of the elder statesmen, the reviewer feels that the time is ripe for major developments along precisely these lines.

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NUCLEIC ACID METABOLISM AND BIOSYNTHESIS^{1,2}

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The appearance of recent reviews covering nucleotide metabolism (1), purine (2, 3) and pyrimidine (4) biosynthesis, and enzymology of nucleic acid catabolism (5) has led to the restriction of the present survey to the area of polynucleotide biosynthesis. Although some overlap is unavoidable, an effort has been made to omit aspects of the subject that are covered elsewhere in this volume, such as protein synthesis, nucleic acid chemistry and structure, and the role of nucleic acids in genetic mechanisms and cell division. The selection of topics, governed in part by space limitations, has been based upon personal interest, and the author has not hesitated to interject notes of criticism as well as purely personal opinion.

BIOSYNTHESIS OF RIBONUCLEIC ACID

ENZYME SYSTEMS ASSOCIATED WITH RNA FORMATION

Polynucleotide phosphorylase.—A problem of major current interest is the nature of the enzymatic pathways leading to synthesis of RNA. Although polynucleotide phosphorylase can catalyze the synthesis of polynucleotides from nucleoside diphosphates [$n(\text{nucleoside diphosphate}) = (\text{nucleoside monophosphate})_n + n(\text{orthophosphate})$], one does not know whether the primary role of the enzyme *in vivo* is synthetic or phosphorolytic. Arguments against a major role for the enzyme in RNA replication are: (a) the ease of reversal in the presence of orthophosphate; (b) distribution limited to bacteria [although suggestions of its presence in higher organisms have appeared (6, 7, 8, 9)]; and (c) product composition reflecting substrate concentration rather than primer composition. Ochoa (10) has recently summarized the reasons for believing that the phosphorylase is involved in RNA synthesis *in vivo*.

A necessary, but not sufficient, requirement for a role in RNA synthesis is the ability to incorporate those purine and pyrimidine analogues that are known to be incorporated *in vivo*. To this end, Lengyel & Chambers (11, 12) synthesized 2-thiouridine-5'-diphosphate and demonstrated that it was

¹ The survey of the literature pertaining to this review was concluded in October, 1960.

² Structures of oligonucleotides or segments of polynucleotides have been abbreviated in conformity with the system used in the *Journal of Biological Chemistry*. The letters, A, G, U, and C represent nucleosides of adenine, guanine, uracil, and cytosine. T represents thymidine; and X or Y, unspecified nucleosides or deoxynucleosides, depending upon context. The letter "p" to the left of a nucleoside represents a 5'-phosphate; to the right, a 3'-phosphate.

utilized by polynucleotide phosphorylase for the synthesis of polythiouridylate and of an RNA-like copolymer with the other normally occurring nucleoside diphosphates. Although the purified phosphorylases of *Azotobacter vinelandii* and *Escherichia coli* are apparently similar in their action, there may be some variation among bacterial species. The phosphorylase isolated from *Micrococcus lysodeikticus* by Olmstead & Lowe (13, 14) had as its sole observable activity the polymerization of adenosine diphosphate to yield polyadenylate. Activity toward cytidine diphosphate, present in the crude extract, was differentially inhibited by iodoacetate, Cu^{++} , and Zn^{++} and completely destroyed by a trypsin digestion step used in the purification of the adenosine diphosphate enzyme. Incubation of the enzyme with a mixture of diphosphates of adenosine, cytidine, guanosine, and uridine yielded only polyadenylate.

In view of its non-discriminating appetite, one of the anomalies associated with the polynucleotide phosphorylase of *Azotobacter* has been its inability to polymerize guanosine diphosphate, except as a copolymer with the diphosphates of adenosine, cytidine, and uridine. Now Singer *et al.* (15) have observed polymer formation from guanosine diphosphate with a highly purified enzyme when small oligonucleotides, such as ApA, pApA, or pApApA, were added as primers. A free 3'-hydroxyl end group was required, and the reaction was shown to proceed by addition of guanylate units to the primer. Chains of up to 30 nucleotide units were formed, chain length being a function of enzyme concentration. Transnucleotidation was noted as a side reaction between oligonucleotides when high enzyme concentrations were used. Exchange between orthophosphate and guanosine diphosphate had been reported as sluggish but apparently proceeds as fast as other nucleoside diphosphate exchanges if optimal concentrations of orthophosphate and Mg^{++} are used (16). Mii (17) has also observed polyguanylate formation by using a high ratio of enzyme to substrate. Polycytidylate stimulated polyguanylate formation; chains were formed of over 100 nucleotide units.

Attempts to elucidate the mechanisms of action of polynucleotide phosphorylase have been facilitated by the use of small chemically defined oligonucleotides. Hilmoie (18) has reviewed the evidence (16) that a free 3'-hydroxyl end group is required for phosphorolysis, and he demonstrated that nucleotides are removed stepwise from the free 3'-hydroxyl end until a dinucleotide or dinucleoside phosphate is left. The ability of small oligonucleotides to overcome the lag phase observed with highly purified *Azotobacter* enzyme was demonstrated by Heppel, Singer & Hilmoie (19, 20). Oligonucleotides as small as ApA with a free 3'-hydroxyl end group abolish the lag phase and are themselves incorporated in the polynucleotide formed. Oligonucleotides with a terminal 3'-phosphate also abolish the lag phase (except for guanosine diphosphate polymerization) but are not themselves incorporated. No oligonucleotide specificity was observed. The mechanism of priming, particularly by oligonucleotides that are not themselves incorporated, is unclear. Beers (21) has utilized the formation of complexes between polynucleotide and acridine orange to study the mechanism of the phos-

phorylase reaction. He has concluded that complex II, which forms at low dye/RNA ratios, is formed by dye reaction at terminal sites on the nucleotide chain, and he has utilized complex II formation to measure concentration of polynucleotide molecules. With this technique, the concentration of polynucleotide molecules at equilibrium was found to be independent of the enzyme concentration, suggesting that the reaction did not proceed by addition of nucleoside diphosphate to any endogenous primer present in the enzyme.

While many synthetic and natural ribonucleic acids are readily phosphorylated by the enzyme to yield nucleoside diphosphates, commercial yeast RNA is resistant. Beers (22, 23) has reported the presence of inhibitors and activators for the polymerization reaction in commercial yeast RNA (and activators in commercial adenosine diphosphate). Heppel *et al.* (19) considered that commercial yeast RNA consisted of relatively short chains which terminated in a 3'-phosphate and accordingly were resistant to phosphorylation or, alternatively, that resistance resulted from the presence of multistranded chains. Ochoa (24) had shown that the complex formed by mixing polyuridylylate and polyadenylylate resisted phosphorylation, and Grunberg-Manago (25) has observed a correlation between resistance and hydrogen bonding. The latter explanation seems applicable to the observation of Singer *et al.* (26) that soluble RNA of rabbit liver is digested by phosphorylase only to the extent of 20 to 30 per cent with no loss of ability to accept activated amino acids and no change in sedimentation coefficient. The authors concluded that the active RNA was totally resistant and that 20 to 30 per cent of inactive RNA was totally degraded.

Incorporation of nucleoside triphosphates: terminal.—The ubiquitous distribution of inorganic pyrophosphatase would favor a synthesis of RNA based upon nucleoside triphosphate condensation: $n(\text{nucleoside triphosphate}) = (\text{nucleoside monophosphate})_n + n(\text{pyrophosphate})$, and $n(\text{pyrophosphate}) \rightarrow 2n(\text{orthophosphate})$. Attempts to demonstrate RNA synthesis in cell-free preparations of mammalian origin led to the observations, summarized in a previous review (1), that nucleoside triphosphates, rather than diphosphates, were incorporated into RNA primarily in a terminal position. Canellakis (27) has reviewed the evidence that cytidine triphosphate and adenosine triphosphate add to soluble RNA in sequence to form RNA-pCpA. This formulation was clearly demonstrated with a chromatographically purified enzyme fraction from liver by Herbert (28), who observed that cytidine triphosphate was added terminally to form RNA-pC and this reaction was followed by the addition of adenosine triphosphate to yield RNA-pCpA. Pyrophosphate was released stoichiometrically and cytidylate and adenylate were incorporated in a 1:1 ratio. In contrast to this 1:1 relationship, Hecht *et al.* (29) reported that, at an optimal pH, two cytidylate units were incorporated per one adenylate, suggesting that the product could be RNA-pCpCpA. These authors demonstrated that the pCpCpA terminal sequence was required for soluble RNA to function in accepting activated amino acids. Confirming evidence for the pCpCpA terminal sequences was provided by the use of C^{14} - and P^{32} -labeled nucleotides with a cytoplasmic

liver homogenate free of nuclei (30). In this case, it was also shown that uridylyate was preferentially incorporated adjacent to a uridylyate residue, as was guanylate, thus suggesting the formation of RNA-pUpUpG (proof that this sequence is terminal was not presented). The heterogeneous nature of cytoplasmic RNA was emphasized by demonstrating that 61 per cent of the incorporated guanylate occurred in a UpG sequence when total RNA was analyzed, whereas, in an RNA fraction eluted from an ECTEOLA column with 0.05 M NaCl, the UpG sequence accounted for 90 per cent of the incorporated guanylate.

In an attempt to purify the enzyme system involved in terminal addition, Herbert & Canellakis (31, 32) obtained three ribonucleoprotein fractions from rat liver cytoplasm. Two of these, designated β and γ , were capable of terminal addition. The enzyme was resolved into separate RNA and protein components, both of which were required for cytidylate or adenylate incorporation. Cytidine triphosphate alone added to the RNA in a terminal position, but in the presence of adenosine triphosphate, two-thirds of the incorporated cytidylate units were non-terminal, suggesting the formation of two distinct RNA types: RNA_I-pC and RNA_{II}-pCpA. Low concentrations of pyrophosphate stimulated the cytidine triphosphate reaction, thus suggesting an exchange with endogenous terminal cytidylate units that result from a reversal of the incorporation reaction. A similar enzyme has been partially purified from *E. coli* by Preiss & Berg (33) and was found to catalyze terminal addition of adenosine triphosphate to purified amino acid-acceptor RNA of *E. coli*. The reaction was stimulated by addition of cytidine triphosphate and by pretreatment of the RNA with venom phosphodiesterase (5 per cent hydrolysis), suggesting formation of RNA-pCpA in conformity with the liver enzyme. Soluble RNA of rat liver cytoplasm has been fractionated on ECTEOLA columns by Goldthwait (34) into two principle components, RNA I eluted with neutral NaCl and RNA II eluted with NH₄OH. Terminal addition of adenylate was observed primarily into RNA I (35). A preparation of RNA I from yeast (36) incorporated adenylate in a terminal position adjacent to a cytidylate unit when incubated with adenosine triphosphate and a rabbit muscle enzyme, even though the RNA I preparation was shown to have no cytidylate end group (37).

The systems derived from rat liver, *E. coli*, and yeast appear to modify at least a fraction of soluble RNA by adding on a terminal -pCpA unit to form RNA species capable of functioning as acceptors of activated amino acids. This may also be the function of an enzyme partially purified from chick embryo extracts which adds adenosine triphosphate terminally to soluble RNA (38). There are, however, other enzyme systems for adding terminal sequences to RNA in which the function of the modified RNA is not known. An example is the suggested formation of the pUpUpG terminal sequence previously mentioned (30). Burdon & Smellie (39, 40) have observed terminal addition of uridine residues. An enzyme that shows specificity for terminal addition of cytidine triphosphate to a specific thymus RNA has been purified 100-fold from calf thymus extracts by Hurwitz and co-workers

(41, 42). It was completely inactive with adenosine triphosphate, and the authors suggest that a separate enzyme may be required for addition of each ribonucleotide. The possibility exists that terminal addition of nucleotides to a "carrier" RNA may be a preliminary step in the biosynthesis of RNA, but definite evidence to support such an hypothesis is not presently available. It is suggestive, however, that incubation of labeled soluble RNA with microsomes results in transfer of some label from soluble RNA to microsomal RNA (43 to 46). Bloemendal *et al.* (46) showed that isotope transfer occurred from a P^{32} -labeled fraction of soluble RNA that was chromatographically isolated to a distinct fraction of microsomal RNA obtained by alkaline elution from an ECTEOLA column. This result tends to discount the possibility that soluble RNA is simply adsorbed to the microsomes. The presence of 5-ribosyluracil phosphate in soluble RNA and its relative absence from microsomal RNA would argue against transfer of complete molecules. If small nucleotide segments are transferred, the question remains open as to whether this process is concerned with protein synthesis, RNA synthesis, or both. Two brief notes have recently suggested an amino acid dependence for adenosine triphosphate incorporation into microsomal RNA (47) and mitochondrial RNA (48).

A matter of considerable interest in the terminal addition reaction is the nature of the soluble RNA acceptor. The soluble RNA of cell cytoplasm is presumably a complex mixture of various species: individual ribonucleic acids for each activated amino acid (29, 49) and other ribonucleic acids which may add terminal nucleotides but are not necessarily concerned with protein synthesis. Despite this heterogeneity, several laboratories have observed regularities in composition. When a polynucleotide chain of the type $pX'pXpX \cdots pXpX''$ is hydrolyzed with alkali, the terminal group X'' is liberated as a nucleoside; the $pX'p$ group at the 5'-phosphorylated end of the chain is released as a mixture of 2',5'- and 3',5'-nucleoside diphosphates; and the internal units are liberated as mixtures of 2'- and 3'-nucleoside monophosphates. The only diphosphate obtained from soluble RNA is guanosine 2'(and 3'),5'-diphosphate (50, 51, 52). A similar uniformity has not been observed for the other end of the chain, which is liberated as free nucleoside, although, in general, the total amount of nucleoside obtained was stoichiometrically equivalent to the amount of guanosine diphosphate. Singer & Cantoni (50) found that adenosine accounted for approximately half of the liberated nucleoside, with cytidine as the next most frequent component from a soluble RNA preparation from rabbit liver. Adenosine was the only nucleoside obtained from *E. coli* soluble RNA by Zillig *et al.* (51). With yeast RNA I, the liberated nucleosides were 62 per cent adenosine and 38 per cent uridine (37). Habermann (53) found mainly cytidine and a small amount of uridine with soluble RNA of mouse liver or pig pancreas. Similar assays performed on their purified soluble RNA preparations, RNA β and RNA γ , by Herbert & Canellakis (52) gave the following percentages: RNA β —adenosine, 60; uridine, 0; guanosine, 24; and cytidine, 10; and RNA γ —adenosine, 23; uridine, 10; guanosine, 51; and cytidine, 14. Despite the heterogeneity implied by

this diversity of terminal groups, both preparations showed the compositional regularities usually associated with double-stranded, hydrogen-bonded structures, i.e., ratios of unity for adenylate to uridylylate plus 5-ribosyluracil phosphate and for guanylate to cytidylate. However, the bulk of evidence, as reviewed by Fresco *et al.* (54), suggests that soluble RNA is a single-stranded molecule with some 60 per cent hydrogen-bonded helical character. These authors have proposed structures based on hydrogen-bonded helices associated with protruding loops and have described the conditions for maximal stability of such structures.

Incorporation of nucleoside triphosphates: non-terminal.—There is at present no definitive evidence for mechanisms by which the terminal addition reaction can lead to net synthesis of RNA. However, early studies on the incorporation of adenosine triphosphate into RNA indicated a second operative pathway, apparently localized in the cell nucleus, by which adenylate units from adenosine triphosphate were incorporated into internal positions in the polynucleotide chain (55, 56). In pursuing this reaction, Edmonds & Abrams (57) purified an enzyme 100-fold from extracts of thymus nuclei. Curiously, this enzyme proved specific for adenosine triphosphate (slight activity was observed with cytidine triphosphate and none with triphosphates of uridine or guanosine). Essentially all of the incorporated adenylate units were in internucleotide linkages. Although the significance of this reaction for RNA synthesis is unknown, several factors are compatible with the concept of individual enzymes for each nucleoside triphosphate acting co-operatively to form RNA: (a) nearest neighbor relationships with the purified enzyme indicate that 99 per cent of the incorporated adenylate units occurred in ApA linkage, whereas with a crude enzyme fraction CpA, ApA, and GpA links were found with approximately equal frequency; (b) in the course of purifying the adenosine triphosphate enzyme, a fraction relatively specific for cytidine triphosphate incorporation was obtained (58); and (c) the small amount of cytidylate incorporated by the adenosine triphosphate enzyme was copolymerized with adenylate units (59). Both enzymes have been separated into protein and polynucleotide components, both of which are required for activity (59).

The incorporation of adenosine triphosphate into both terminal and internucleotide positions has been observed with a soluble fraction of chick embryo organs (38, 60). Incorporation was stimulated slightly by other nucleoside triphosphates, singly or in combination, and by soluble RNA. Manganese was shown to enhance internucleotide incorporation. Anionic polymers, including DNA, stimulated at low concentrations and inhibited at higher levels, whereas the reverse was found with spermidine and cadaverine. Incorporation of uridine (presumably uridine triphosphate) into both terminal and internucleotide positions has been reported with soluble extracts of Ehrlich ascites cells (39, 40, 61). The reaction was stimulated somewhat by other nucleoside triphosphates and, surprisingly, by microsomal RNA.

As is indicated elsewhere in this review, the nucleus is a major site of RNA synthesis within the cell, possibly the only one. In any case, if the nature of the proteins synthesized in the cytoplasm is governed by genetic informa-

tion residing in DNA in the nucleus, the current hypothesis seems reasonable: an agent for transfer of such information must be formed in the nucleus, this agent possibly being a form of RNA synthesized so as to replicate nucleotide sequences of DNA. Rich (62) has recently demonstrated in this connection that it was possible to form a double-stranded hydrogen-bonded structure between a polyribotide chain and a polydeoxyribotide chain. One might then hope to be able to demonstrate a nuclear enzyme for RNA synthesis that is dependent upon the presence of DNA and requires all four nucleoside triphosphates in analogy with DNA polymerase. Some progress in this direction has been forthcoming. Weiss has demonstrated that a DNA-containing particulate fraction obtained from rat liver nuclei catalyzed the incorporation of cytidine triphosphate into RNA only if all four nucleoside triphosphates were present (63, 64). The reaction was inhibited by ribonuclease and by deoxyribonuclease. With an *E. coli* preparation, Stevens (65) reported an incorporation of adenosine triphosphate into RNA which was dependent upon the presence of all four nucleoside triphosphates and was inhibited by ribonuclease and deoxyribonuclease. The newly formed RNA could be separated from the bulk of inactive RNA by gradient centrifugation and was shown to be localized in the small ribosome fraction, a fraction which incorporated P^{32} most rapidly *in vivo* in *E. coli*. Also working with *E. coli*, Hurwitz *et al.* (66) obtained a thirtyfold purification of an enzyme fraction which incorporated uridine triphosphate into RNA. All four nucleoside triphosphates were required, as was DNA. DNA from various sources was equally effective. Evidence was cited for more or less random incorporation of the labeled nucleotide within the polynucleotide chain.

These experiments, which seem to implicate DNA in RNA synthesis, are indeed exciting and seemingly reasonable in view of current theories of information transfer from nucleus to cytoplasm. However, until the enzymes involved are adequately purified, their interpretation must take cognizance of possible pitfalls. Thus, DNA might function indirectly, for example, as an inhibitor of an endogenous ribonuclease; and even the extra nucleoside triphosphates could, in some circumstances, function to maintain the labeled precursor at the triphosphate level or provide phosphate ion if it should be required.

SITES OF RNA SYNTHESIS WITHIN THE CELL

The fact that labeled precursors appear in nuclear RNA much more rapidly than in cytoplasmic RNA has been well-established since the original observation of Marshak (67). The introduction of radioautographic methods has led to the demonstration that within the nucleus the nucleolus is strongly labeled by RNA precursors. The problem of whether nuclear RNA is a precursor of cytoplasmic RNA and whether chromatin-associated RNA is a precursor of nucleolar RNA, or vice versa, continues to be an active field of investigation.

Injection of tritiated uridine into *Drosophila melanogaster* by Zalokar (68) resulted in exclusive labeling of nuclear RNA within minutes. After one hour cytoplasm was also heavily labeled but not so intensely as the nucleus.

Zalokar (69) has reported an ingenious investigation of RNA-labeling in *Neurospora* hyphae exposed to short pulses of tritiated uridine. Centrifugation of the hyphae at 50,000×g resulted in stratification of the organelles. From bottom to top, the layers corresponded to glycogen, ergastoplasm (microsomes), mitochondria, nuclei, cytoplasmic sap, vacuoles, and fat. After a one minute pulse with uridine, the cells were incubated in unlabeled uridine. At 4 min., label was mostly nuclear; at 60 min., mainly microsomal. The author concluded that 99 per cent of the labeled cytoplasmic RNA originated in the nucleus. Incorporation studies with tritiated cytidine and human amnion and HeLa cells in tissue culture (70, 71) have been interpreted as indicating transfer of RNA from nucleus to cytoplasm. Harris (72), on the other hand, has observed that 8-azaguanine and β -2-thionylvaline inhibited adenosine and cytidine uptake more extensively for cytoplasmic RNA than for nuclear RNA. He concluded there was no necessary coupling between the two ribonucleic acids. Attempts have been made (73, 74) to measure RNA transport from nucleus to cytoplasm by incubating labeled nuclei in unlabeled cytoplasm. With adenine-8- C^{14} , release of nuclear RNA is a two-phase process independent of energy supply but stimulated by ATP and citrate. Previous results with orotate-labeled nuclei (74, 75) which indicated more rapid release from recently labeled nuclei were not confirmed with adenine (73).

Another approach to this problem has been the study of RNA synthesis in enucleated cells. Prescott (76) has suggested that the finding of precursor incorporation into RNA in enucleate halves of *Amoeba proteus* by Plaut & Rustad (77) resulted from viable yeast cells in the food vacuoles. With amoeba starved for 72 hr., no incorporation of uracil, orotate, or adenine could be demonstrated with enucleate fragments. Similar experiments with *Acanthamoeba* sp. (78), which can be grown in sterile nutrient medium, confirmed the negative result with enucleate fragments where starvation was not a factor. Enucleate halves of human amnion cells have also been reported to be unable to synthesize RNA (79). The report (80) that enucleate fragments of *Acetabularia mediterranea* are capable of net RNA synthesis was contradicted by Richter (81), who observed no net synthesis. In contrast to their earlier findings, a subsequent report from Brachet's laboratory (82) agrees that there is no net over-all synthesis of RNA in enucleate fragments. However, it was observed that during regeneration of the enucleate fragment, C^{14} -adenine, orotate, and bicarbonate were incorporated into RNA and that all of the RNA of the cell was in a large granule fraction which consisted largely of chloroplasts. The authors conclude that the nucleus may be essential for the maintenance of microsomal and soluble RNA but that chloroplastic RNA is synthesized independently in the cytoplasm. This view has been summarized in a review of the subject by Brachet (83). The localization of RNA in the chloroplasts of *Acetabularia* is in contrast with *Euglena* where 50 to 60 per cent of cytoplasmic RNA is in the microsomes and approximately 10 per cent in chloroplasts (84). Brawerman & Chargaff (85) observed that chloroplast regeneration was associated with isotope incorporation into

the RNA of all cell organelles, and they cited evidence for the formation of a new and distinct RNA.

Within the nucleus, the interrelationship of nucleolar and extranucleolar RNA has been the subject of widely disparate results. The subject has been discussed by Sirlin (86) who cited evidence for two types of RNA within the nucleolus which differ in rate of labeling and localization. Fitzgerald & Vinijchaikul (87) observed the incorporation of tritiated cytidine in acinar and islet cells of rat pancreas. Expressing data in terms of the fraction of cells in the microscope field that shows grain count greater than background over the given cell structure, highest labeling was nucleolar at 0.5 hr., nuclear at 4 hr., and cytoplasmic at 24 hr. The authors conclude that their data are compatible with RNA transport from nucleolus to nucleus to cytoplasm. Radioautographic studies of tritiated uridine incorporation into the salivary gland chromosomes of *Smittia* sp. indicated that RNA was synthesized in the nucleolus adjacent to the chromosomes with a subsequent spread through the nucleolus and along the chromosome (88). Amano & Leblond (89) have attempted to determine RNA transport relationships by means of specific activity curves, in which specific activity is defined as the ratio of grain counts to intensity of toluidine blue stain. The tissues studied were liver and pancreas of mice injected with H^3 -cytidine. Although early labeling was greater in chromatin than in the nucleolus, the fact that the cytoplasmic curve intersected only the nucleolar curve led the authors to conclude that RNA synthesis occurred independently in chromatin and nucleolus, the latter migrating to the cytoplasm and the former undergoing independent metabolism and breaking down *in situ*.

Taylor & Woods (90) used a somewhat different technique to determine specific activity changes in salivary gland cells of *Drosophila repleta* after a short period of feeding on adenine-8- C^{14} . Early labeling was maximal in the nucleolus, and after 4 hr. RNA of nucleolus and chromatin had similar specific activities which diminished in parallel fashion. Another approach to the problem, devised by Perry (91) involved selective destruction of the nucleolus of HeLa cells by a microbeam of ultraviolet light 2.2 μ in diameter. Such irradiation, compared to a control irradiated adjacent to the nucleolus, resulted in a drop of 30 per cent for nuclear RNA uptake of tritiated cytidine and 65 per cent for cytoplasmic RNA. The author postulated that RNA was transported from nucleolus to nucleus to cytoplasm with an independent synthesis in the extranucleolar nucleus, which was also transported to cytoplasm. The data are cited as being incompatible with transport from nucleus to nucleolus.

Several reports have appeared which strongly contradict the possibility of primary RNA synthesis as an exclusively nucleolar function. With human amnion cells exposed to tritiated cytidine, it was observed that after 5 min. 28 per cent of the nuclear grains were over the nucleolus, whereas after 10 min. the figure was 45 per cent (92). It was concluded that RNA was transported from chromosomes to nucleolus. Comparable studies at longer time intervals led to similar conclusions for other mammalian cells in tissue culture

(93). Pelling (94) observed that tritiated uridine was taken up preferentially by RNA-rich bands in the salivary gland chromosomes of *Chironomus tentans* with a diffusion of isotope from the nucleolar organizer out into the nucleolus. It was concluded that many chromosomal sites are involved in RNA synthesis, the bulk of the RNA being produced in a few very active loci largely associated with puffs, Balbiani rings, and nucleolar organizers. The nucleolus is looked upon simply as a transit station. Indirect evidence which de-emphasizes the role of the nucleolus comes from the observation that benzamide, which inhibits production of moulting hormone by the prothoracic gland of *Drosophila*, also inhibits adenine incorporation into nuclear RNA but not into nucleolar RNA (95). With amphibian embryos, adenine incorporation into RNA is most rapid around the nuclear membrane (96).

Harris has seriously questioned whether nucleolar RNA differs at all from nuclear RNA in rate of formation and, in fact, whether any RNA within the nucleus is a precursor of cytoplasmic RNA (72, 97, 98). RNA turnover was demonstrated by loss of isotopic label when the unlabeled precursor, adenosine, was added to the medium. Nucleolar and nuclear counts dropped in parallel fashion with no evidence of transfer to cytoplasm. However, Taylor (99) has pointed out that Harris' data (98) are not necessarily incompatible with a nuclear site of origin of cytoplasmic RNA.

The one firmly established fact in this general area of conflicting claims and data is the observation that nuclear RNA is labeled more rapidly than cytoplasmic RNA. There is a strong implication that a fraction of nuclear RNA may be a precursor of at least some cytoplasmic RNA, but absolute proof is not yet at hand. It seems likely that some of the uncertainty is associated with limitations of technique. Thus, Venkataraman (100) has observed that absolute alcohol extracts 25 to 30 per cent of the RNA from a tissue homogenate precipitated with cold trichloroacetic acid. In a tracer experiment, it was the most highly labeled RNA that was extracted. The implication of this for preparations for autoradiography is obvious. The widespread use of tritiated precursors introduces uncertainties which are not always adequately evaluated. Difficulties in interpreting radioautographs which stem from the short range of tritium β -particles [half thickness of 0.3 μ in water (101)] have been pointed out by Woods & Schairer (102). It has also been emphasized by Oliver & Lajtha (103) that the amount of tritium needed to get good radioautographs may result in radiation damage to sensitive biochemical reactions. Krause & Plaut (104) have in fact observed an apparent radiation effect of H^3 -thymidine on DNA synthesis which did not exist when C^{14} -thymidine was used as the tracer. Finally, the possibility is always present that incorporation of a precursor may represent terminal addition rather than synthesis of a new polynucleotide molecule.

BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID

ORIGIN OF DEOXYRIBONUCLEOTIDES

Two possible pathways leading to deoxyribose have been known for some time: (a) the condensation of acetaldehyde with D-glyceraldehyde-3-phosphate to form 2-deoxyribose-5-phosphate catalyzed by the enzyme deoxy-

ribose-5-phosphate aldolase (105); and (b) the direct reduction of ribosides or ribotides to the corresponding deoxyribose derivative (106). Although Boxer & Shonk (107) have indicated that the deoxyribose-5-phosphate aldolase is particularly active in tumor extracts, studies on the distribution of C^{14} from labeled precursors in ribose and deoxyribose formed in *E. coli* (108) and in mammalian tissues (109) have indicated that a ribose compound is a precursor of deoxyribose or that they are both formed from a common precursor. Pricer & Horecker (110) have purified the aldolase of *Lactobacillus plantarum* and found it specific for acetaldehyde but not for D-glyceraldehyde-3-phosphate. Other suitable reactants were L-glyceraldehyde-3-phosphate, D-glyceraldehyde, D-erythrose, D-erythrose-4-phosphate, D-ribose-5-phosphate, glycolaldehyde phosphate, and D-erythrose.

Attempts to demonstrate conversion of nucleosides or nucleotides to the corresponding deoxyribose compounds in cell-free systems have had some success during the past year. Reichard & Rutberg (111) observed deoxycytidine nucleotide formation from cytidine-5'-phosphate labeled with H^3 or P^{32} in soluble extracts of *E. coli* B. The reaction had an absolute requirement for adenosine triphosphate and Mg^{++} and, after treatment with Dowex 2, for reduced triphosphopyridine nucleotide. In short-term experiments with deoxycytidine mono-, di-, and triphosphate carriers, the highest specific activity was observed in the deoxycytidine diphosphate fraction. The authors suggested that adenosine triphosphate phosphorylated cytidine-5'-phosphate to cytidine diphosphate, which was converted to deoxycytidine diphosphate by reduced triphosphopyridine nucleotide. Moore & Hurlbert (112, 113) have reported a similar reduction of cytidine-5'-phosphate in extracts of Novikoff hepatoma also requiring adenosine triphosphate, Mg^{++} , and reduced triphosphopyridine nucleotide. When the extract was precipitated at pH 5, both the supernatant solution and the precipitate were required for deoxycytidylate formation. The same reaction in extracts of Ehrlich ascites carcinoma cells and calf thymus was observed by Abrams *et al.* (114). In this case, however, reduced diphosphopyridine nucleotide was required. The reduction is not specific for pyrimidine nucleotides; Reichard (115) has demonstrated a similar reaction with guanosine-5'-phosphate in chick embryo extracts. An earlier description of reduction of various ribosides and ribotides to the corresponding deoxyribose compounds in extracts of *Salmonella typhimurium* (116) was shown by Reichard & Rutberg (111) to be an artifact.

Aside from the suggestion that the reaction takes place at the diphosphate level, little is yet known about the steps involved in the reduction of nucleotides to deoxynucleotides. Reichard *et al.* (117) have observed that deoxycytidylate formation was strongly inhibited by the triphosphates of deoxyadenosine, deoxyguanosine, and thymidine. That this inhibition might also occur *in vivo* was indicated by Morris & Fischer (118) who observed that growth of neoplastic cell lines requiring thymidine was inhibited by excess thymidine and the inhibition was overcome by small amounts of deoxycytidine. The possibility that an intermediate step in the reduction involved the formation of an oxygen bridge between C-2 of the pyrimidine and C-2' of the ribose was investigated with chick embryo minces by Reichard (119)

and with *E. coli* by Pizer & Cohen (120). With both these systems, $O^2:2'$ -cyclonucleosides were metabolically inert. It is of interest that the pyrimidine arabinosides, spongouridine and spongocytidine, which bear a steric resemblance to the deoxyribosides, can meet the uracil requirement of *E. coli* mutants (120).

A role for vitamin B_{12} in the biosynthesis of deoxyribonucleotides has long been suspected from microbiological studies which have shown that the B_{12} requirement of *Lactobacillus leichmannii* could be replaced by deoxyribonucleosides. Several recent reports have attempted to demonstrate by means of labeled precursors that B_{12} was necessary for deoxyribose formation in this organism. Wacker *et al.* (121) grew *L. leichmannii* with randomly labeled guanosine- C^{14} in a medium fortified either with deoxycytidine or with B_{12} . Only in the latter case was the deoxyribose of DNA-deoxyguanosine significantly labeled. The same result has been reported by Manson (122). In a similar experiment, Floyd & Whitehead (123) used randomly labeled uridine- C^{14} in a medium containing either deoxyguanosine or B_{12} plus guanine. Only in the latter case did C^{14} appear in the deoxyribose of DNA-thymidine. In interpreting these experiments, it must be borne in mind that the positive results need not have been related to the presence of B_{12} but rather that the negative results could have stemmed from the addition of deoxynucleosides to the medium. It is not unreasonable to suppose that, under some conditions, added deoxyriboside would repress deoxyribose formation (117) or that any deoxyribose formed would be diluted with unlabeled deoxyribose as a result of the trans-N-deoxyribosidase present in *L. leichmannii*. This criticism does not apply to the demonstration by Spell & Dinning (124) that B_{12} was required for the appearance of C^{14} from ribose-1- C^{14} in the DNA-deoxyribose. It is strange, however, that DNA and RNA had the same specific activities despite the availability of deoxycytidine in the medium. Itzhaki (125) has shown with randomly labeled glucose- C^{14} and rat thymus cells that C^{14} in DNA appears only in the deoxyribose of purine deoxyribosides, and it is not unlikely that the same phenomenon is represented in this experiment with *L. leichmannii*.

Attempts to demonstrate an influence of B_{12} on deoxyribose synthesis in higher organisms have been unsuccessful. Using suspensions of B_{12} -deficient chick bone marrow cells containing deoxyuridine in the medium, Dinning & Young (126) observed that B_{12} stimulated incorporation of formate- C^{14} into DNA-thymidine. Bolinder & Reichard (127), working with chick embryo minces from B_{12} -deficient hens, reported that conversion of pyrimidine ribonucleotides to the corresponding deoxyribonucleotides was normal in B_{12} deficiency and not stimulated by B_{12} . A depression was observed, however, for deoxyuridine conversion to DNA-thymidine but was overcome by addition of B_{12} . Both papers conclude that the primary effect of B_{12} on deoxyribonucleotide synthesis in the chicken is on the introduction of the thymine methyl group. It has been pointed out (127) that the involvement of B_{12} in deoxyuridylylate synthesis is not ruled out if one assumes that one of the enzymes has an extremely high affinity for B_{12} . Evidence has been obtained

favoring (128, 129) and opposing (123) a role of B_{12} in converting formate- C^{14} to the methyl group of thymine in *L. leichmannii*.

One aspect of deoxyribose metabolism in which interest continues concerns the enzyme trans-N-deoxyribosidase. A preparation purified elevenfold from extracts of *Lactobacillus helveticus* by Roush & Betz (130) catalyzed transfer of deoxyribose from purine or pyrimidine deoxyribosides to guanine, adenine, hypoxanthine, 8-azaguanine, 6-mercaptopurine, thymine, uracil, and cytosine. The reaction was inhibited by trishydroxymethyl amino-methane buffer. An enzyme purified fiftyfold from *Lactobacillus acidophilus* R-26 by Marsh & King (131) had similar properties. Uric acid, 2,6-diaminopurine, and 8-azaxanthine failed to serve as deoxyribose acceptors, and transfer from thymidine to adenine was inhibited competitively by 6-azathymidine. Minghetti (132), using a partially purified preparation from *L. leichmannii*, observed both a phosphate-dependent and a phosphate-independent transfer reaction. The latter reaction required the purine or pyrimidine of the deoxyribose donor to have a hydroxyl group in the 6-position. 5-Bromocytosine served as a good deoxyribose acceptor, but 2-mercaptopuracil and 2-mercaptopcytosine were poor, and 2-methylmercaptopuracil was inactive. A trans-N-deoxyribosidase also occurs in *E. coli* but is absent from *Lactobacillus delbrueckii*, *Lactobacillus casei*, yeasts, molds, beef, rat, chicken, and crayfish (130, 131). In view of its limited distribution, its general significance in deoxyribose metabolism is dubious.

ENZYME SYSTEMS ASSOCIATED WITH DNA FORMATION

In contrast to the uncertain status of the enzyme processes involved in RNA synthesis, no alternatives to the DNA polymerase reaction observed by Kornberg and co-workers are known at present. The work by this group with an enzyme purified some 4000-fold from extracts of *E. coli* has been reviewed recently by Lehman (133) and, in his Nobel lecture, by Kornberg (134). For DNA formation the enzyme requires the presence of Mg^{++} , some DNA "primer," and all four of the deoxyribonucleoside triphosphates (adenine, guanine, cytosine, and thymine). The properties of this enzyme strongly implicate it as an essential element in biological replication of DNA. Thus, since it is possible to synthesize 20 times more DNA than is added as "primer," it has been shown that the newly formed DNA duplicates the "primer" in terms of viscosity, sedimentation coefficient, molecular weight, and composition.

Kornberg (134) has emphasized that the "primer" DNA acts as a template, with the enzyme incorporating only those deoxyribonucleotides that will form a hydrogen-bonded pair with a purine or pyrimidine on the template. In line with this suggestion is the observation (135) that the enzyme will utilize analogues of the natural purines or pyrimidines only when they replace their specific counterparts.

Of particular significance in relation to hydrogen bonding and the Watson-Crick model (136) are some recent experiments on nearest neighbor relationships in the newly synthesized DNA. If a deoxynucleoside triphos-

phate labeled in the α -phosphate with P^{32} , which may be designated as $X\text{-}P^*\text{-PP}$, is incorporated into DNA, the labeled phosphate will be bonded to the 3'-position of an adjacent deoxynucleoside residue, designated as Y, in the DNA strand: $\dots pYp^*Xp\dots$. When the DNA is hydrolyzed with micrococcal deoxyribonuclease and splenic phosphodiesterase, the 5'-phosphate ester bonds are cleaved and the P^{32} is released as Yp^* , thus providing a measure of the relative frequency of the dinucleotide sequence YpX in the DNA chain. If X' represents a deoxynucleoside that can form a hydrogen-bonded pair with X (such as thymidine if X is deoxyadenosine) and Y' a corresponding hydrogen-bonded "mate" of Y, then, if replication involves the formation of hydrogen-bonded double strands, the frequency of occurrence of the sequence YpX will be equal to the frequency of $X'pY'$ for antiparallel strands or to $Y'pX'$ for parallel strands. Preliminary reports of such experiments (134, 137) carried out with several different deoxyribonucleic acids as primers have indicated: (a) all 16 possible dinucleotide sequences are always found; (b) the distribution of dinucleotide frequencies is characteristic of each individual DNA; and (c) replication does involve base-pairing of adenine with thymine and guanine with cytosine in two antiparallel strands.

Shapiro & Chargaff (138) have pointed out that, in addition to base-pairing based on hydrogen-bond formation, another selection factor must operate in DNA replication. They utilized limited acid hydrolysis to determine frequencies of pyrimidine nucleotide sequences of the type purine-pyrimidine-purine ("solitary" pyrimidine nucleotides) and purine-pyrimidine-pyrimidine-purine ("coupled" pyrimidine nucleotides). With deoxyribonucleic acids of wheat germ and rye germ, the distribution of 5-methyldeoxycytidine was such that it could not have been incorporated randomly in place of deoxycytidine. Even more striking was the effect of replacing thymine by 5-bromouracil in the DNA of a thymine-requiring auxotroph of *E. coli* (139). Although base analysis indicated no changes except replacement of one-third of the thymine residues by 5-bromouracil, the fraction of "solitary" pyrimidine nucleotides was doubled. The frequencies of "solitary" pyrimidine nucleotides in native DNA in moles per 100 moles of DNA phosphorus were thymidylate, 3.82, and deoxycytidylate, 1.78; whereas for 5-bromouracil-containing DNA they were thymidylate, 3.01; 5-bromodeoxyuridylate, 2.96; and deoxycytidylate, 3.60. The authors emphasize that 5-bromouracil has not simply replaced thymine but has induced major changes in nucleotide sequences. They suggest an exclusion principle in replication such that any deoxynucleotide will accept certain neighbors and reject others. It will be of interest to see this type of experiment repeated with purified DNA polymerase with a direct determination of nearest neighbor sequences.

It has been observed that some incorporation of deoxynucleoside triphosphates occurs even if all four of the required triphosphates are not present. A "limited reaction" involves the addition of one or a few units to the end of the DNA chain bearing a free 3'-hydroxyl (140), and it has been stated that this process is controlled by hydrogen-bonding requirements (134). A new type of polymer can be formed after a long lag period when the enzyme is incubated with deoxyadenosine triphosphate plus thymidine triphosphate

(133, 141). This deoxyadenylate-thymidylate polymer, which is of high molecular weight and contains equivalent amounts of adenine and thymine, will prime its own synthesis, thus abolishing the usual lag period. Nearest neighbor relationships indicate a perfectly alternating sequence of thymidylate and deoxyadenylate units (142). A corresponding deoxyguanylate-deoxycytidylate polymer can be formed from deoxyguanosine triphosphate and deoxycytidine triphosphate after a lag period (142). In this case, nearest neighbor relationships indicate that the polymer is a mixture of polydeoxyguanylate and polydeoxycytidylate chains. The perfect orderliness of these unnatural polymers, where one might have expected random incorporation, is indeed surprising.

Reports that the same, or a very similar, DNA polymerase occurs widely in mammalian tissues, particularly those with high mitotic indices, continue to appear (143 to 148). The best characterized of these has been purified fiftyfold from calf thymus extracts by Bollum (149) and still has a specific activity several hundredfold lower than the *E. coli* enzyme which it resembles in general properties. Bollum (149, 150, 151) has emphasized that native thymus DNA is completely inactive as a primer and becomes active only after being heated (10 min. at 100°) or treated with acid or alkali. The single-stranded DNA obtained from phage ϕ X-174, however, was active as a primer without prior denaturation. An apparent priming action with the calf thymus enzyme has been observed by using small oligodeoxynucleotides (152). With a homologous series of polythymidylates (from pTpTpT to pTpTpTpTpTpTpT) as primers, a "limited reaction" was observed with deoxyadenosine triphosphate or with deoxycytidine triphosphate in which a single product was formed which differed little in mobility on paper chromatograms from the oligodeoxynucleotide primer. With thymidine triphosphate or deoxyguanosine triphosphate, however, a series of products of decreasing mobility was formed, the most highly polymerized material (of zero mobility) increasing with time. This situation is reminiscent of polynucleotide phosphorylase in which oligonucleotide intermediates are not observed except when small oligonucleotides are used as primers (20). Although adequate data are not yet at hand, one has the impression that the polymerase can catalyze two basic types of reaction, one in which oligonucleotides can serve as true primers with units being added to the primer to produce longer chains, and one in which the polynucleotide "primer" is actually a template directing the formation of replicates of itself. Only in the latter case need hydrogen bonding between base pairs function to determine the nature of the product.

Of particular significance in the replication process is the observation that the thymus enzyme, which exhibits very little nuclease activity at the optimum pH for DNA synthesis, has an absolute requirement for single-stranded DNA. It has also been stated (134) that the best *E. coli* preparations are active only with heat-denatured or single-stranded DNA. One would expect on this basis that the polymerase would be limited in its action to one replication of single-strand primer molecules, thereby forming an equal number of double-stranded hydrogen-bonded DNA molecules. It re-

mains to be seen whether the *E. coli* enzyme, once it is freed of nucleases, will promote only a doubling of primer DNA rather than the twentyfold increase now observed.

Concerning the mechanism of replication, Atwood (153) has recently pointed out that simultaneous replication of each strand (154) as the double-stranded DNA unwinds was unlikely, since different mechanisms would have to be evoked for each strand: addition of deoxynucleoside triphosphate to 3'-hydroxyl groups for one strand and addition to 5'-phosphate for the other. Atwood suggested that replication would be sequential, one chain splitting off as the other is replicated, or semisequential, both chains undergoing simultaneous replication from opposite ends. An alternative possibility is the complete conversion of the double-stranded DNA to single strands which would then be replicated by the polymerase. Such a mechanism would accord with the observation (155), based upon shifts in ultraviolet inactivation spectra, that infection of *E. coli* B with T2r⁺ phage results in a change in the character of the phage DNA to single-strandedness 3 min. after infection and then back to double-strandedness at 11 min. Studies on the interaction of mammalian DNA with Pb⁺⁺ have suggested that single- and double-stranded forms may exist as an equilibrium mixture (156), although this seems in contradiction to the total inactivity of thymus polymerase with native DNA.

An inhibitory control mechanism for DNA synthesis has been suggested in several preliminary reports. Polymerase and thymidine kinase activities, which are low in extracts of normal rat liver, increase to high values in regenerating liver (157). That this increase might represent removal of an inhibitor rather than new synthesis is suggested by the observation of Weissman *et al.* (158) that extracts of normal liver inhibit the polymerase and kinase activities of regenerating liver extracts. A partially purified polymerase was uninfluenced by extracts of proliferating tissues but strongly inhibited by extracts of non-proliferating tissues, such as liver or kidney. The thermolabile, non-dialyzable inhibitor was not a deoxynucleoside triphosphate phosphatase. A different type of inhibition was found by Reichard *et al.* (117), who observed that the reaction sequence, cytidine-5'-phosphate → deoxycytidine triphosphate → DNA, was strongly inhibited by traces of deoxyadenosine triphosphate or deoxyguanosine triphosphate. The inhibition was localized at the step in which the ribonucleotide was converted to a deoxyribonucleotide. This observation probably accounts for several other reports of inhibition of DNA synthesis by deoxyadenosine (159, 160, 161). This type of inhibition bears investigation in relation to the observed decrease in DNA synthesis in regenerating liver *in vivo* at a time when polymerase activity is still increasing (157).

INHIBITION OF DNA SYNTHESIS BY IONIZING RADIATION

Ever since the initial observation of von Euler & Hevesy (162) that x-rays decreased the incorporation of P³²-orthophosphate into DNA of Jensen sarcoma, there has been a growing impression that the primary effect of ionizing

radiation on cells was interference with DNA synthesis. However, Kelly (163), in a review of this subject in 1957, concluded that DNA synthesis was radioresistant and that the observed inhibition was a result of changes in cell population and inhibition of mitosis. Howard (164) has also emphasized that mitotic delay and cell death could account for most of the observations of impaired DNA synthesis. To avoid the possibility of changing cell populations, Harrington (165) has irradiated U-12 fibroblasts growing in tissue culture with 500 r and observed an immediate cessation of cell division. DNA synthesis was reduced at a later time; it was maximally depressed 18 to 24 hr. after irradiation. Since the reduction in DNA specific activity when H^3 -thymidine was incorporated paralleled the reduction in number of cells incorporating thymidine, Harrington suggested that those cells synthesizing DNA were doing so at a normal rate and that the primary effect of irradiation was on cell division. Cells unable to complete mitosis were unable to enter the subsequent phase of DNA replication.

In contrast to these results, a considerable body of evidence exists that the over-all process of DNA synthesis is radiation-sensitive. Howard & Pelc (166) have indicated that the interphase period of the cell cycle could be subdivided into a postmitotic presynthetic period, G_1 ; a period of DNA synthesis, S; and a postsynthetic premitotic period, G_2 . Their results, and particularly those of Lajtha *et al.* (167), have indicated that, in addition to the mitotic block induced by radiation, relatively low doses of x-rays delivered to G_1 cells interfered with their ability to enter the DNA synthetic period, S, and higher doses diminished the rate of DNA synthesis during the S period. In apparent contradiction to the results of Harrington (165), Lajtha *et al.* (168) have found that irradiation of human bone marrow cells in tissue culture during the S period lowers the amount of label (C^{14} -formate) incorporated into DNA per cell but does not affect the percentage of labeled cells. These authors have demonstrated that the dose-response curve during the S period is biphasic and consists of two exponential components, S_1 (to 500 rads) and S_2 (500 to 1300 rads). The suggestion was made that S_1 represents a relatively radiation-sensitive period of DNA precursor synthesis and that the more resistant S_2 represents damage to the DNA template. Essentially the same result was obtained with Ehrlich ascites carcinoma cells and H^3 -thymidine, although the difference in slope between S_1 and S_2 was not so marked as with bone marrow cells (169). A biphasic dose-response curve has also been noted for P^{32} incorporation into rat thymus DNA measured 2 hr. after irradiation [Ord & Stocken (170)]. The break between radiation-sensitive and radiation-insensitive response occurred at 200 r of total body x-irradiation. The authors suggested that the initial sensitive phase corresponded to inhibition of DNA precursor synthesis, particularly phosphorylation of nucleotides in the nucleus, during the G_1 period, whereas the second more resistant phase represented progressive template damage during the S period. In confirmation of this argument, Creasey & Stocken (171) found that doses as low as 44 r completely inhibited the ability of isolated nuclei from proliferating tissues to increase their acid-labile phosphate content

when shaken in air at 0°. Myers (172) has cast considerable doubt upon the significance of this observation by demonstrating that the net synthesis of diphosphorypyridine nucleotide by several rat tissues following nicotinamide injection is uninfluenced by 1500 r of total body irradiation. Since diphosphopyridine nucleotide synthesis is exclusively a nuclear function (173) and since adenosine triphosphate is required for this synthesis (174), it is apparent that even large doses of x-rays do not interfere with the availability of nucleoside triphosphates in the nucleus. Reports continue to appear (175, 176) on effects of irradiation on nucleoside triphosphate levels or turnover, but the inhibitions observed are generally negligible.

In line with their suggestion that higher radiation doses depress DNA synthesis as a result of template damage, Ord & Stocken (177), using the technique of Bendich *et al.* (178), observed a change in chromatographic profile for rat thymus DNA as a result of 1000 r total body irradiation when the DNA was passed through an ECTEOLA column. Irradiation produced a large increase in the peak eluted with 2 M NaCl in 0.1 M NH_4OH at the expense of the tightly held component eluted with 0.5 M NaOH.

The technique of quantitative autoradiography after a short exposure to H^3 -thymidine following irradiation has been used by Sherman and co-workers (179, 180) to demonstrate radiation effects on cells in the phase of DNA synthesis. Their results indicated a significant drop in the rate of DNA synthesis during the S period for cells of mouse intestinal epithelium and hair follicles as a result of 400 to 800 rads of total body x-irradiation.

One of the major difficulties in determining whether inhibition of DNA synthesis is a primary result of irradiation or secondary to factors such as mitotic delay or cell death is the randomness of the cell population with respect to the mitotic cycle. This difficulty can be obviated by the use of cell populations that divide in synchrony. With a synchronously dividing culture of *Tetrahymena pyriformis*, Gardella & Servello (181) observed that DNA synthesis was linear throughout the 90 min. growth cycle. Irradiation with 60,000 rads at any time during the second growth cycle resulted in an immediate inhibition of 80 to 90 per cent of P^{32} uptake into DNA. The cells subsequently recovered, and, after a mitotic delay, DNA synthesis was normal in the next generation. These results suggest an inhibition of DNA synthesis as a primary event.

Among mammalian tissues, regenerating liver approximates a synchronously growing population. Holmes (182) has reviewed the evidence that a wave of DNA synthesis occurs 18 to 24 hr. after partial hepatectomy and is followed some hours later by mitosis and cell division. A dose of 450 r delivered 12 hr. after hepatectomy delayed DNA synthesis and the subsequent onset of mitosis for 10 hr., whereas 2000 r was required to inhibit DNA synthesis once it had begun. By using radioautographic methods with H^3 -thymidine and microspectrophotometric methods for DNA content of individual hepatocytes during regeneration, Looney (183) found the mean time for DNA replication, i.e., the S period of the cell cycle, to be 8 hr. An x-ray dose of 3000 r delivered 17 hr. after hepatectomy, when DNA synthesis had already begun, directly affected the rate of DNA synthesis and increased the replica-

tion time from 8 hr. to 13 hr. (184). The results with regenerating liver confirm the impression obtained from more heterogeneous mitotic systems: ionizing radiations inhibit DNA synthesis independently of their effects upon cell division, with a period of high sensitivity in the presynthetic interphase followed by a period of lower sensitivity once DNA synthesis has begun.

Several efforts have been made to correlate x-ray inhibition of DNA synthesis in regenerating liver with the enzymes involved in DNA synthesis. Beltz & Applegate (185) exposed partially hepatectomized rats to 1500 r at 12 to 13.5 hr., and at 25 hr. They assayed homogenates for DNA synthesizing activity in a system fortified with DNA primer and the triphosphates of deoxycytidine, deoxyadenosine, and deoxyguanosine. They observed a 90 per cent inhibition of H^3 -thymidine incorporation into DNA. In a similar study, Bollum *et al.* (186) irradiated rats with 375 to 1500 r at 6 hr. after partial hepatectomy (during the G_1 period) or at 16 hr. (during the S period). Liver extracts prepared at 30 hr. were assayed for thymidine kinase and DNA polymerase. Both enzyme activities were strongly depressed by irradiation at 6 hr. but uninfluenced by irradiation at 16 hr., even though the 16 hr. irradiation produced a marked inhibition of DNA synthesis *in vivo*. The authors suggested that irradiation early in regeneration blocked synthesis of the enzymes required for DNA formation, whereas late irradiation, when the enzymes were already present, damaged the DNA template. In assaying polymerase activity after late irradiation, van Lancker (187) depended upon endogenous primer and observed a marked inhibition of H^3 -thymidine incorporation. The assumption of template damage would make this observation compatible with those of Bollum *et al.* (186). Okada (188) has compared normal DNA with irradiated DNA as primer in an *in vitro* polymerase assay. The results were somewhat equivocal; irradiation of the primer inhibited H^3 -thymidine incorporation but not H^3 -deoxycytidylate incorporation.

Several studies have appeared recently which may relate to the radiation-sensitive events during the presynthetic interphase period. To the extent that RNA is involved in protein synthesis, the radiation induced block in the synthesis of thymidine kinase and DNA polymerase during the G_1 period could reflect an action on RNA synthesis. Payne *et al.* (189) observed several years ago that total body irradiation depressed P^{32} incorporation into liver nuclear RNA but not into cytoplasmic RNA. Similarly, with Ehrlich ascites carcinoma cells *in vitro*, doses of 750 to 3000 r have no effect on incorporation of uracil-2- C^{14} into cytoplasmic RNA but repress incorporation into DNA and nuclear RNA in a parallel fashion (190). Fractionation of nuclear RNA into RNA I (soluble in 1 M NaCl) and RNA II (insoluble in 1 M NaCl) was carried out by Klouwen (191) with thymus and liver from rats exposed to 700 r and subsequently injected with P^{32} -orthophosphate. With liver, P^{32} incorporation into RNA I was inhibited; with thymus, both RNA I and RNA II were depressed. In a similar study with regenerating liver, Welling & Cohen (192) observed that P^{32} incorporation into nuclear RNA reached a peak during the presynthetic G_1 period and then dropped sharply as DNA synthesis began. Irradiation with 700 r, if carried out within 4 hr. after hepatectomy, resulted in a 40 to 60 per cent inhibition of P^{32} incorporation into

both RNA I and RNA II. Further evidence suggesting a radiation-sensitive period of protein synthesis prior to DNA formation has been reported by Billen (193, 194). DNA synthesis was inhibited by using *E. coli* 15T₋ and blocking protein synthesis with chloramphenicol or by amino acid deprivation. Upon removal of the block in protein synthesis, DNA was again formed after a lag, but x-irradiation prior to the beginning of DNA synthesis completely prevented its restoration.

As indicated in the foregoing paragraphs, several authors have proposed that the inhibition observed when cells were irradiated during the period of DNA synthesis was a result of template change. Hems (195) has recently summarized the nature of the radiation-induced changes observed with solutions of DNA. Both purine and pyrimidine bases are destroyed, the yield being dependent upon the presence of oxygen. Purines suffer opening of the imidazole ring to form 4-amino-5-formamidopyrimidines (196), whereas pyrimidines are attacked at the 4,5-double bond to form a mixture of *cis* and *trans* 4-hydroxy-5-hydroperoxypyrimidines [Eckert & Monier (197)]. Attack on the purine and pyrimidine bases is accompanied by a hyperchromic effect, presumably as a result of hydrogen-bond breakage (198). Hems (195) has estimated that one sugar-phosphate backbone bond is broken (in an oxygen independent process) for four bases destroyed and that a biologically significant dose of 1000 rads will destroy one base in 10⁴, a not insignificant figure even if one assumes a certain degree of protection by other nuclear constituents. That destruction of bases may result in significant functional damage is suggested by the observation of Englander *et al.* (199) that main chain breakage accounts for only 40 per cent of the inactivation of tobacco mosaic virus upon irradiation. Extrapolation from effects of irradiation on DNA in solution to effects in the cell has posed many problems. Fisher *et al.* (200) have been able to detect changes in viscosity of dilute solutions (in 1.43 M NaCl) of crude DNA-nucleoprotein preparations with x-ray doses as low as 10 r. Much higher doses were required in more concentrated solutions. When undissociated DNA-nucleoprotein particles, as prepared by the method of Doty & Zubay (201), were used, Emmerson *et al.* (198) were unable to detect any damage to DNA with doses of 10⁵ rads. They proposed that the cell nucleus was sensitive to irradiation only during those phases of the mitotic cycle in which DNA had shed its protein coat. Another effect of x-rays on DNA-nucleoprotein was proposed by Hagen (202); i.e., irradiation labilized metal ion linkages between DNA and protein. The amount of DNA extractable upon shaking with phenol in the presence of a low concentration of trichloroacetic acid was increased following irradiation. This effect, however, developed slowly, so that it is not likely to be involved in the immediate effect upon DNA synthesis.

To summarize, present evidence suggests that ionizing radiations suppress mitotic division and independently interfere with DNA synthesis. For the latter action, sensitivity is greatest in the interphase period before actual synthesis begins.

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LIPID METABOLISM^{1,2}

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It has become increasingly, even painfully, evident that progress in the field of lipid metabolism has grown so rapidly during the past few years that the ability of reviewers to survey more than a few small areas in a vast terrain has been outstripped. Thus, no apologies are made for the fact that this review is not comprehensive; were it so, we could not have written it, very few could find time to read it, and certainly the *Annual Review of Biochemistry* could not have allotted sufficient space to print it.

TRIGLYCERIDE ABSORPTION

Previous studies by Borgstrom (14, 24, 25) and others have demonstrated that ingested triglycerides are partially hydrolyzed in the intestinal lumen and that a mixture of fatty acids and mono-, di-, and triglycerides is absorbed by the mucosal cells and transported to the intestinal lymphatics, where the absorbed lipid appears entirely as triglyceride in the chylomicrons (24). From previous work, the concept has also arisen that the chylomicrons consist essentially of tiny droplets of triglyceride, which owe their stability in an aqueous environment to adherent molecules of specific lipoproteins (109, 139) which are synthesized by the intestine (140) and added to the triglyceride droplets during their transit from the intestinal lumen to the lacteals.

Events occurring within the mucosal cell during triglyceride absorption have been the subject of recent investigation, and a new dimension has been added by the application of electron microscopy to this problem (5, 36, 122, 170). Sections of rat intestinal mucosa have been examined by both light and electron microscopy prior to and at intervals subsequent to the ingestion of corn oil. The pictures obtained indicate that at least part, and perhaps all, of the absorbed fat is removed from the intestinal lumen as small droplets which enter between the microvilli of the striated border of the epithelial cells by the process of pinocytosis. From here the droplets enter the tubular spaces of the endoplasmic reticulum and, perhaps aided by peristaltic contraction of smooth muscle fibers within the villi, travel through the epithelial cells to their lateral borders, where the triglyceride is discharged into the intercellular spaces in a form morphologically indistinguishable from chylomicrons. Since the intercellular spaces are continuous with the lymphatics,

¹ The survey of the literature pertaining to this review was concluded in October, 1960.

² The following abbreviations are used: CoA for coenzyme A; CDP for cytidine diphosphate; CTP for cytidine triphosphate; NADP for nicotinamide-adenine dinucleotide phosphate; NADPH₂ for nicotinamide-adenine dinucleotide phosphate, reduced form.

no further barrier to entry into the circulatory system is evident. If the lumen of the endoplasmic reticulum is regarded as being continuous with the extracellular phase, then the entire pathway of triglyceride absorption may be extracellular. However, this does not preclude the possibility of metabolic interaction between the lipid droplets within the endoplasmic reticulum and the cytoplasmic components of the epithelial cells; indeed, it is highly likely that this does occur. The possible general significance of the picture of triglyceride absorption becomes evident when it is considered that lipid entry into liver (5, 133) and adipose tissue (10, 138) cells may be accomplished by a very similar mechanism.

In addition to, or ancillary to, mass transport of lipids by pinocytosis, intraluminal and intracellular hydrolysis and synthesis of triglycerides occur, and it has been estimated (133) that 50 per cent of ingested triglyceride glycerol is released during digestion and absorption. Coniglio & Cate (38) have examined the fate of ingested 1-C¹⁴-palmitic acid and tripalmitin-carboxyl-C¹⁴ in the intestinal lumen and intestinal wall of rats. During the first 3 to 6 hr. following tripalmitin feeding, the principal radioactive constituents of the intestinal contents were triglyceride and palmitic acid, whereas in the intestinal wall most of the isotope was present as triglyceride, with appreciable amounts also in the phospholipid and fatty acid fractions. Following the ingestion of 1-C¹⁴-palmitic acid, the major form present in the intestinal contents was palmitic acid, but in the intestinal wall the isotope was present principally as triglycerides and, to a lesser extent, as phospholipid and fatty acid. The latter finding is consonant with the observation (43, 171) that everted intestinal sacs *in vitro* not only absorb C¹⁴-palmitic acid from the mucosal surface but convert it to triglyceride, which is expelled in the form of chylomicrons from the serosal side. From this and earlier work, it is apparent that intestinal mucosal cells are capable of triglyceride synthesis from fatty acids and that this represents a pathway of absorption of fatty acids present in the gastrointestinal tract. The chemical pathway by which triglycerides are formed (37) appears to be identical with that of liver (182). However, intestinal mucosal cells (30) do not seem to contain glycerokinase, which probably accounts for the fact that ingested glycerol-C¹⁴ is not converted to glyceride-glycerol (132). Evidence has been obtained (43) which suggests that bile acid conjugates, such as taurocholate, in some manner promote intracellular fatty acid esterification during absorption.

Conflicting reports have appeared concerning the extent of dilution of dietary triglycerides by endogenous lipid (20, 29, 31, 49) during digestion and absorption.

TRIGLYCERIDE METABOLISM

Although the principal immediate fate of chylomicron triglyceride is still unsettled, important contributions have been made to this subject recently. Previous evidence (24, 59) suggested that triglyceride hydrolysis is important and perhaps essential for assimilation of chylomicron glycerides. However, it

is now clear that this view must be modified somewhat. Electron-microscopic studies (5) have revealed that lipid-rich particles morphologically indistinguishable from chylomicrons appear in the hepatic sinusoids following a fatty meal. These can be traced into the pericellular spaces of Disse, which they apparently enter through pores in the sinusoidal endothelial cytoplasm. From this site, the lipid droplets enter the hepatic parenchymal cells by pinocytosis and form cytoplasmic lipid inclusions in a manner strikingly similar to that observed in intestinal epithelial cells (122). Such a mechanism of entry is compatible with data (24, 133, 159) obtained from isotopic tracer studies which suggest that plasma triglycerides having their origin in dietary fat are incorporated into hepatic triglyceride without detectable hydrolysis. Moreover, Rodbell (138) has reported that the initial uptake of triglycerides by adipose tissue, a tissue in which pinocytosis has been observed (10), apparently does not involve lipolysis, but that complete hydrolysis and re-esterification seem to be prerequisites for the storage of triglycerides entering this tissue. Rodbell has suggested that, in the initial step, chylomicrons may enter the peripheral cytoplasm of these cells by pinocytosis but that hydrolysis and re-esterification must precede transfer to the central lipid globule. The identity of the enzyme responsible for hydrolysis of triglycerides entering adipose tissue remains uncertain. Although it has been widely assumed that the heparin-activated lipoprotein lipase purified by Korn (96) from heart muscle and from avian adipose tissue may be the active enzyme, this is by no means certain.

It has been reported that rat, but not human (4), adipose tissue contains lipoprotein lipase which can be liberated by incubation with heparin *in vitro* (35) and that the amount of enzyme extractable from this tissue is decreased by starvation, heparin treatment, or alloxan diabetes (86, 124). Moreover, the rate of hydrolysis of added chyle triglycerides is greater by adipose tissue from carbohydrate-fed rats than from fasted animals (142); in addition, the rapid hydrolysis of chyle triglyceride by adipose tissue from fed rats is inhibited by protamine, pyrophosphate, or high salt concentration, whereas the slower process in starved tissue is unaffected (141, 142) by these inhibitors of lipoprotein lipase (96). In view of the fact that net free fatty acid release from endogenous triglycerides is greater in "starved" than in "fed" tissue (142), the above observations suggest that an adipose tissue enzyme that has properties similar to those of avian lipoprotein lipase (96) may play the postulated role. However, it should be pointed out that such an enzyme has not yet been obtained in purified form from mammalian adipose tissue. Lynn & Perryman (115) have reported the extensive purification and properties of a mammalian adipose tissue lipase which does not require a lipoprotein or heparin as activators and which contains no detectable carbohydrate. The physiological function of this enzyme, whether in uptake of exogenous fats or hydrolysis of endogenous glycerides, has not yet been ascertained.

Active phagocytosis of emulsified fats and chylomicrons, as well as their

subsequent oxidation, has also been reported to occur in macrophages (44) and leukocytes (90). Moreover, extraction of plasma triglycerides by cardiac muscle *in vivo* has been observed (13), although it is not known whether this occurs by pinocytosis. It seems possible that either pinocytosis or the similar process of phagocytosis may represent a general mechanism for the entry of triglycerides into cells.

As previously mentioned, triglyceride hydrolysis and resynthesis appear to be prerequisite for fat storage in adipose tissue. Furthermore, adipose tissue itself actively synthesizes fatty acids from non-lipid precursors (24, 77) and these fatty acids also can be stored. Finally, there is continual breakdown and resynthesis of stored triglyceride within adipose tissue cells (24, 133). Since this tissue, like intestinal mucosa, is unable to utilize glycerol (155), it is dependent for fatty acid esterification upon a continuous supply of α -glycerol phosphate from glycolysis (191).

FATTY ACID SYNTHESIS

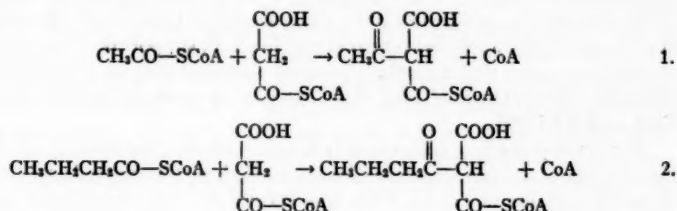
In 1958, malonyl CoA was identified as an intermediate in the conversion of acetyl CoA to higher fatty acids by fractionated avian liver extracts (27, 175). More recently, the status of this compound as a fatty acid precursor and as an obligatory intermediate in fatty acid synthesis has been examined in a variety of tissues. Moreover, information has been sought concerning the pathway by which the malonyl moiety is converted to higher fatty acids.

Ganguly (60) has reported that 2-C¹⁴-malonyl CoA is converted to higher fatty acids by soluble extracts from a variety of animal tissues, including supranrenal fat, mammary gland, and liver. In each case, malonyl CoA was found to be far more efficient than acetyl CoA as a fatty acid precursor. In addition, it was found that conversion of acetyl CoA to fatty acids by a bovine liver extract was accelerated sevenfold by addition of a purified enzyme which catalyzed the carboxylation of acetyl CoA to malonyl CoA. This suggests that carboxylation of acetyl CoA is rate-limiting for its conversion to higher fatty acids via malonyl CoA.

Reactions concerned in the transformation of malonyl CoA to higher fatty acids by fractionated soluble extracts (61) of avian liver have been investigated by Wakil & Ganguly (176, 177). The enzyme preparation employed catalyzed the conversion of malonyl CoA to higher fatty acids (principally palmitate) in the presence of NADPH₂^a and acetyl CoA. Substitution of butyryl CoA or octanoyl CoA for acetyl CoA resulted in the incorporation of these acyl units into higher fatty acids. Although both acetyl CoA and

^a Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH₂), for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP) for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH₂), for triphosphopyridine nucleotide, reduced form (TPNH).

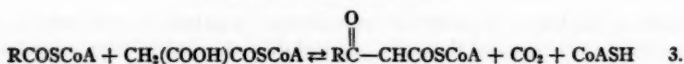
malonyl CoA were required for optimal rates of palmitate synthesis, isotopic tracer studies revealed that only one-eighth of the carbon atoms of palmitate were derived from acetate, whereas the remainder had their origin in malonate. On the basis of this evidence, the authors suggest that elongation of the carbon chain of fatty acids occurs by condensation of one molecule of a saturated acyl CoA with a molecule of malonyl CoA:



According to this scheme, the two methyl terminal carbon atoms of each fatty acid molecule are derived directly from acetyl CoA and the remainder have their origin in malonyl CoA. Horning, *et al.* (87), employing a partially purified adipose tissue system which synthesizes higher fatty acids from malonyl CoA in the presence of acetyl CoA and NADPH₂, have provided additional support for this concept. These workers observed that substitution for acetyl CoA of various branched-chain saturated acyl CoA derivatives resulted in formation of those higher branched-chain fatty acids anticipated if only one acyl unit were incorporated into the final product. For example, substitution of isocaproyl CoA for acetyl CoA resulted in the formation of a product tentatively identified as 14-methyl pentadecanoic acid.

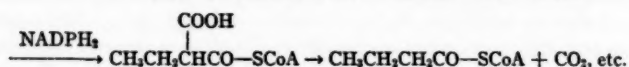
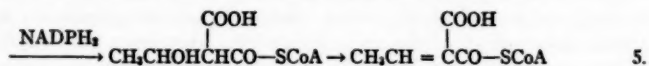
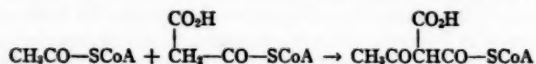
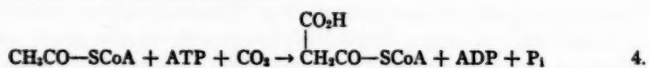
As pointed out above, Wakil (177) has postulated that condensation of a saturated acyl CoA derivative with malonyl CoA initially yields a substituted malonyl CoA derivative (Equation 1 above). In support of this, Steberl, Wasson & Porter (158) have reported that fractionated avian liver extracts incubated with malonyl CoA and either acetyl or butyryl CoA accumulate products which they have identified chromatographically as the expected β -ketodicarboxylic acid derivatives. However, Vagelos & Alberts (173) have presented evidence for an alternative mechanism for the condensation of various acyl CoA derivatives with malonyl CoA. They observed that the activity of a purified enzyme from *Clostridium kluyveri* catalyzes an exchange reaction between malonyl CoA and $\text{HC}^{14}\text{O}_2^-$ which is dependent upon the addition of boiled cell extract. The active component was isolated and identified as caproyl CoA. It was also found that any of a series of straight-chain fatty acyl CoA derivatives tested were active in stimulating this exchange; however, acetyl CoA and butyryl CoA were less effective than longer-chain fatty acyl CoA derivatives.

Such an exchange strongly suggests the occurrence of a concerted condensation decarboxylation reaction which yields the β -ketomonocarboxylic acid intermediate directly:



The K_m values for the series of compounds that stimulated this exchange were measured and found to decrease significantly with increasing chain lengths over the range from butyryl CoA to myristyl CoA. The broad specificity of the enzyme for acyl CoA compounds and the increasing affinity that it exhibits toward the substrate as the chain length is lengthened also suggests that it might play a role in fatty acid synthesis. In support of this suggestion, the authors report that a partially purified exchange enzyme from *C. kluyveri* catalyzes the synthesis of long-chain fatty acids from malonyl CoA, acetyl CoA, and NADPH₂.

From the preceding discussion, it is apparent that the identity of the initial product of the condensation of malonyl CoA with saturated acyl CoA derivatives remains in doubt. Even less certain are the identities of further compounds that may be intermediates in the conversion of malonyl CoA to fatty acids. Although the CoA derivatives of fatty acids of intermediate chain length may serve as precursors of higher fatty acids, they have not been shown to accumulate as intermediates in the conversion of malonyl CoA to palmitate in avian liver extracts. Furthermore, it has been reported (177) that the CoA esters of β -keto, β -hydroxy, or α,β -unsaturated acids are not metabolized in avian liver extracts, which are capable of fatty acid synthesis from malonyl CoA. To account for these observations and for evidence suggesting that malonyl CoA is an obligatory intermediate in the conversion of acetyl CoA to higher fatty acids, Wakil has proposed the following sequence as a pathway for fatty acid synthesis:



It is apparent that if $\text{CD}_3\text{CO-SCoA}$ were used as the initial reactant, one would predict that each molecule of palmitate formed by this pathway would contain only three atoms of deuterium and that these would be bound exclusively to the terminal methyl carbon atom. Actually, 15 years ago, Rittenberg & Bloch (137) measured the incorporation of $\text{D}_3\text{C-C}^{18}\text{OOH}$ into the liver and carcass fatty acids of intact rodents. It was found that approximately one deuterium atom accompanied each atom of C^{18} incorporated into the

saturated fatty acids. Moreover, it was established by degradation that deuterium was uniformly distributed along the carbon chain of oleic acid, which was also isolated. These results may be interpreted to mean either that the above scheme is incorrect or that the formation of malonyl CoA as an intermediate in the conversion of acetate to fatty acids is a minor pathway in intact animals.

Wakil (178) has continued his studies implicating biotin in fatty acid synthesis. The synthesis of palmitate from acetyl CoA by enzyme fractions from avian liver is inhibited by avidin, and this inhibition can be prevented by the prior addition of *d*-biotin. The enzyme fraction concerned with the carboxylation of acetyl CoA to form malonyl CoA contains an unusually high concentration of biotin, which accompanies the enzyme through subsequent purification steps. It appears likely that biotin participates in this carboxylation reaction in a manner similar to that described by Lynen (113) for the carboxylation of β -methylcrotonyl CoA to β -methylglutaconyl CoA by a biotin-containing enzyme system from bacteria.

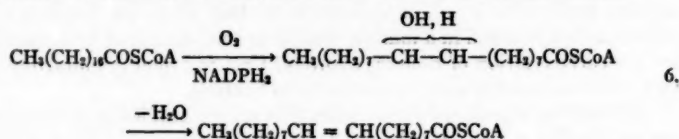
It should be mentioned at this point that several studies have been carried out concerning the effect of biotin deficiency on the rate of fatty acid synthesis. Gram, Okey & Geiger (65) observed a decreased rate of conversion of acetate-2- C^{14} into the liver but not the carcass lipids of biotin-deficient rats which were compared with pair-fed controls. However, Guggenheim & Olson (67) in a similar experiment noted no alteration in the rate of synthesis of hepatic fatty acids. Of perhaps more physiological significance are the studies of Curran (39), who fed D_2O to biotin-deficient and control animals and estimated the rate of fatty acid synthesis from deuterium incorporation data. No change in the synthesis of carcass fatty acids and an increased synthesis of hepatic fatty acids were observed in the biotin-deficient animals. With yeast, on the other hand, biotin-deficiency resulted in striking impairment of fatty acid synthesis which could be reversed readily by addition of biotin to the medium (21).

Brady *et al.* (28) have reinvestigated the inhibition of fatty acid synthesis produced by arsenite in a partially purified enzyme system from rat liver, which requires malonyl, CoA, acetyl CoA, and $NADPH_2$ for long-chain fatty acid synthesis. Although mercaptans such as mercaptoethanol and 2,3-dimercaptopropanol (BAL) stimulated fatty acid synthesis, low concentrations of these thiols were also necessary to demonstrate a highly inhibitory action of arsenite. In high concentrations, BAL reversed the arsenite inhibition. From this and other considerations, it was concluded that closely juxtaposed sulfhydryl groups participate in the reductive process of fatty acid synthesis.

Synthesis of long-chain fatty acids from acetyl CoA by rat liver mitochondria and mitochondrial acetone powder extracts has recently been described by Wakil, McLain & Marshaw (179). ATP, $NADPH_2$, $NADH_2^+$, and a dialyzable heat-stable cofactor are essential for activity; neither bicarbonate nor malonyl CoA are required. It seems possible that this enzyme system

may closely resemble the system reconstructed by Seubert *et al.* (152) from purified mitochondrial enzymes of fatty acid β -oxidation and NADP-ethylene reductase (102).

Fatty acid interconversions.—Bloomfield & Bloch (21) have reported in detail studies of the desaturation of palmitic to palmitoleic acid and stearic to oleic acid by extracts of anaerobically grown yeast. They have directly demonstrated that fatty acids participate in this reaction as the CoA thioesters. The conversion of palmityl CoA to palmitoleyl CoA is catalyzed by a single particulate yeast fraction and requires only a reduced pyridine nucleotide and molecular oxygen. Since these requirements suggest a similarity to hydroxylation reactions, it was postulated that the mechanism of desaturation involves formation and dehydration of the hydroxyl intermediate:



Recently, Lennarz & Bloch (108) have obtained strong support for this hypothesis by showing that tritium-labeled 9-hydroxy stearic acid was converted to olefinic acid by crude yeast homogenates in the presence of ATP, NADPH₂, and CoA. Furthermore, both 9- and 10-hydroxy stearic acids can replace oleic acid as growth factors for anaerobically grown yeast.

PHOSPHOLIPID AND TRIGLYCERIDE SYNTHESIS

The biosynthesis of phospholipids and triglycerides has been critically discussed by Kennedy (94) in Volume 26 of this series. Reproduced in Figure 1 is the scheme he has recently proposed to summarize the pathway for the conversion of glycerol to phospholipids and triglycerides (123).

Phosphatidic acid.—There now appears to be at least two pathways for the synthesis of phosphatidic acid. In addition to the reaction between α -glycerolphosphate and CoA thiol esters of fatty acids originally described by Kornberg & Pricer (97) (Figure 1, Reaction 2), Hokin & Hokin (85) have recently reported that phosphatidic acid is also formed from α,β -diglyceride and ATP (Figure 1, Reaction 7). In brain microsomal preparations they found that the incorporation of P³² from ATP³² into phosphatidic acid was highly dependent upon the presence of added α,β -diglyceride. This activity, for which they propose the name diglyceride kinase, was stimulated most strongly by diglyceride from cabbage lecithin; diglyceride from brain lecithin and 1-palmityl 2-oleyl diglyceride were much less effective. α -Glycerolphosphate was shown not to be an intermediate in this reaction. Bradbeer & Stumpf (26) have observed a similar pathway for phosphatidic acid formation in mitochondria from peanut cotyledons. Both preparations also catalyze the phosphorylation of monoglycerides by ATP to form a compound that appears to be monoacyl phosphatidic acid (26, 84). Since monoglyceride

stimulates the incorporation of label from ATP³² into phosphatidic acid, Hokin & Hokin (84) suggest that monoacyl phosphatidic acid is in turn converted to phosphatidic acid, presumably by the acylating enzyme of Kornberg & Pricer. This stimulation was not observed in the peanut system where the acylating enzyme is known to be absent.

Although phosphatidic acids are actively synthesized by each pathway, it has not been possible to directly demonstrate their natural occurrence in animal tissues until recently. Hokin & Hokin (83) labeled brain phospholipids with P³² and recovered labeled material, which was indistinguishable from

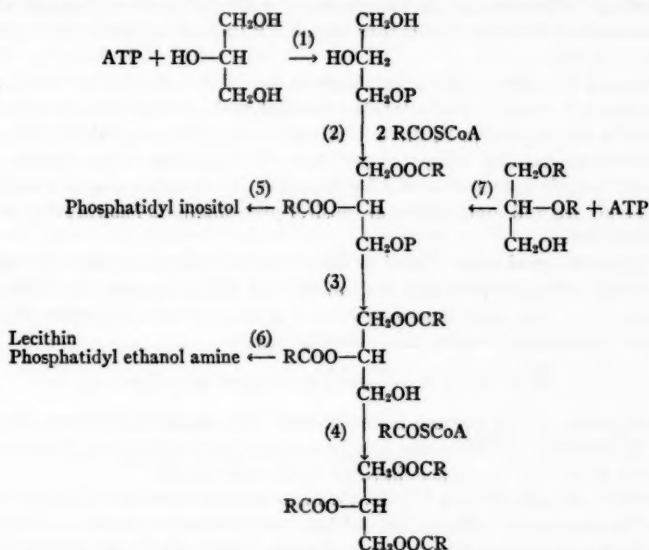
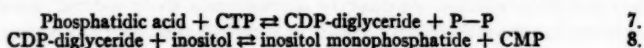


FIG. 1. Pathway for the conversion of glycerol to phospholipids and triglycerides.

cabbage phosphatidic acid. More recently, Hubscher & Clark (88) have reported the isolation and identification of phosphatidic acid from mammalian liver.

Inositol phosphatides.—Paulus & Kennedy (123) have published a complete report of their studies of inositol monophosphatide biosynthesis by a guinea pig microsomal system. P³²-labeled L- α -glycerolphosphate is converted to inositol monophosphatide without the loss of P³² that is observed during its conversion to other phospholipids or triglycerides. Since phosphatidic acid is an effective precursor and therefore appears to be an intermediate, the pathway for inositol monophosphatide synthesis must differ from the others in that it does not proceed through the α,β -diglyceride (see Fig. 1). CTP and free inositol are specifically required for the incorporation of

L- α -glycerolphosphate or phosphatidic acid. When α -glycerolphosphate and CTP are incubated in the absence of inositol, an ether-soluble compound accumulates, which appears to be identical with cytidine diphosphate dipalmitin. Finally, the synthesis of inositol monophosphatides from synthetic cytidine diphosphate diglycerides and inositol has been demonstrated. The pathway from phosphatidic acid therefore can be represented as follows:



Earlier, Agranoff *et al.* (3) had proposed a similar pathway, except that they considered CDP choline rather than CTP to be involved in the formation of CDP-diglyceride.

Paulus & Kennedy (123) have observed that their preparation catalyzed an exchange between tritium-labeled inositol and inositol monophosphate which does not appear to be directly related to the *de novo* synthesis of inositol monophosphatides. They suggest that this exchange accounts for the nucleotide requirements observed by Agranoff (3). A comprehensive review of the structure and metabolism of inositol phospholipids has recently been published (78).

Triglyceride synthesis.—The close interconnection between the enzymatic synthesis of phospholipids and neutral fat was first suggested by Weiss & Kennedy (181) with their demonstration of a net synthesis of triglyceride in chicken liver preparations by the following reaction:



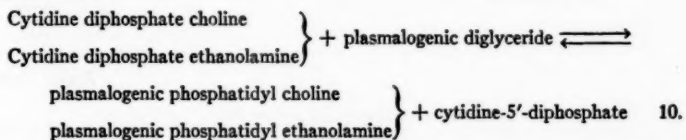
The properties of this system have recently been described in some detail (182). α, β -Diglyceride, of course, had been recognized earlier as an immediate precursor of lecithin and phosphatidyl ethanolamine (94).

Further support for this relationship has recently been provided by evidence that saturated and unsaturated fatty acids occupy similar positions in the lecithin and triglyceride molecule. Tattre (169) and Hanahan *et al.* (74) have reinvestigated the positional specificity of attack by phospholipase A and have found that, contrary to previous concepts (39, 73), the beta-position of lecithin is the site from which free fatty acid is released. It therefore must be concluded that the unsaturated fatty acids of lecithin are esterified with the β -hydroxyl of glycerol. Although the position of saturated and unsaturated fatty acids in triglycerides from various sources is not quite as definite, in most cases unsaturated fatty acids also predominate at the beta-position (74, 119, 146). However, the fact that certain phospholipids and triglycerides share a common precursor does not necessarily imply that the fatty acid composition of the two must be identical. Weiss and co-workers (182) have found that the enzymes catalyzing triglyceride and lecithin synthesis exhibit different specificities for α, β -diglycerides, and they therefore suggest that each pathway can effect a selection from the mixture of α, β -diglycerides normally available. However, the re-evaluation of this distribution of fatty

acids in lecithin certainly makes the demands for such a selection much less rigorous than it might have appeared previously.

Plasmalogens.—It is now well-established that plasmalogens structurally resemble conventional glycerophosphatides, such as phosphatidyl choline, very closely except that one fatty acid group is replaced in the plasmalogens by a fatty aldehyde, which is probably linked to the glyceryl residue by a vinyl ether bond (130). Discrepancies between various laboratories over the position of the vinyl ether linkage are now apparently resolved. In view of the re-defined specificity of phospholipase A, the results of Rapport & Franzl (129) and Gray (66) must be interpreted as suggesting that the vinyl ether bond is in the alpha-position of the glycerol moiety. Marinetti *et al.* (116) and De-buch (46) had arrived earlier at this conclusion.

The enzymatic synthesis of phosphatidyl choline involves a reaction between D- α,β -diglyceride and cytidine diphosphate choline. This prompted Kiyasu & Kennedy (95) to investigate whether the synthesis of choline plasmalogens occurs by a similar reaction between CDP-choline and an α,β -diglyceride in which one ester linkage has been replaced by a vinyl ether bond. This compound, for which the name plasmogenic diglyceride was proposed, was isolated from the products of a lecithinase D-catalyzed hydrolysis of phosphatidyl choline-rich fraction from beef heart. When incubated with rat liver particles and CDP choline or CDP ethanolamine, the following reactions occurred:



Lands (100) has obtained evidence that the fatty acid portion of the lecithin molecule turns over without a corresponding synthesis and breakdown of the remainder of the molecule. He has recently detected an enzyme system from rat liver microsomes which catalyzes the acylation of lysolecithin by fatty acyl CoA to form lecithin (101). The newly formed bond is attacked by phospholipase A and therefore is a β -ester. This enzyme, in combination with phospholipase A, which is widely distributed in animal tissues, can account for an independent turnover of at least one fatty acid moiety of lecithin.

KETONE BODY METABOLISM

At least two enzymic pathways for the formation of free acetoacetate from acetoacetyl CoA have now been described. Lynen and co-workers (112) have previously proposed that the reaction sequence shown in Figure 2 is the major pathway for acetoacetate formation.

Drummond & Stern (50; see also 162), however, have prepared an enzyme from beef liver which apparently catalyzes a more direct hydrolysis of aceto-

acetyl CoA. Although their enzyme preparation contained β -hydroxy- β -methyl-glutaryl CoA cleavage enzyme (Reaction 2 in Figure 2), complete inhibition of the latter enzyme by iodoacetamide treatment did not decrease the rate of acetoacetate formation. These authors therefore suggest that the formation of free acetoacetate need not occur through the intermediate formation of hydroxymethyl glutaryl CoA but may occur by direct deacylation. Furthermore, the livers of various species contained quantities of the hydrolytic enzyme sufficient to account for the rate of acetoacetate formation by these tissues. The relative contributions of these pathways to cellular metabolism is uncertain. Segal & Menon (149) have obtained convincing evidence that the formation of acetoacetate in normal liver proceeds almost exclusively by the direct deacylation of acetoacetyl CoA. This was demonstrated by incubating C^{14} -acetyl CoA and unlabeled acetoacetyl CoA with a liver

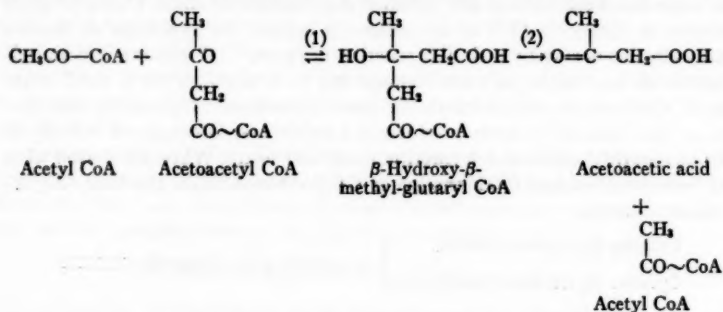


FIG. 2. Major pathway for acetoacetate formation.

mitochondrial preparation, which accounts for the bulk of the acetoacetate-forming activity of liver; the acetoacetate formed contained very little radioactivity. If it were formed by the hydroxymethyl glutaryl CoA pathway, acetoacetate would contain the same specific activity as acetyl CoA. With the aid of isotopic tracers and some rather dubious assumptions, Hird & Symons (82) have concluded that the hydroxymethyl glutaryl CoA pathway occurs in rat liver and sheep omasal tissue, but the method employed would not have detected the direct deacylation pathway had it occurred.

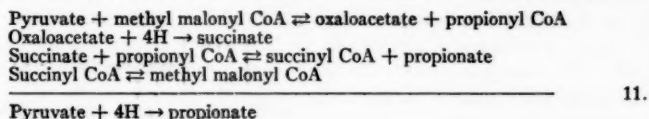
The mechanism responsible for diabetic ketosis has also received attention. It has become increasingly clear (98, 99, 103) that the hepatic overproduction of acetoacetate cannot, as was formerly assumed (180), be attributed simply to a deficiency of oxalacetic acid with consequent accumulation of acetyl CoA. Rather convincing circumstantial evidence exists which suggests that ketosis may result from uncoupling of oxidative phosphorylation in liver mitochondria when the intracellular concentration of free fatty acids increases; evidence for this viewpoint has recently been summarized (103). On the other hand, increased acetoacetyl CoA deacylase activity has been found

in the hepatic mitochondria of diabetic rats (148, 150). Moreover, it has been reported (188) that the activities of both hydroxymethyl glutaryl CoA condensing enzyme and hydroxymethyl glutaryl cleavage enzyme (Reactions 1 and 2 of Figure 2) are increased twofold in the livers of alloxan diabetic rats, whereas the concentration of hydroxymethyl glutaryl CoA is only slightly increased. Although it is apparent that this enzymic alteration may be important, it seems unlikely that these observations can account for ketosis. It has been known for some time (111, 180) that both normal and diabetic liver slices produce acetoacetate at very great, but approximately equal, rates when incubated with butyric or octanoic acids. Were the steps catalyzed by the enzymes of the hydroxymethyl glutaryl CoA pathway rate-limiting, one would anticipate that acetoacetate production from these short-chain acids would be greater in diabetic liver than in the normal tissue.

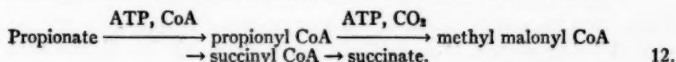
In mammalian liver, free acetoacetate is reversibly reduced by a NAD-linked⁸ enzyme, D-β-hydroxybutyric dehydrogenase. Preparation and properties of this enzyme have been described by Lehninger and co-workers (106).

PROPIONIC ACID METABOLISM

Swick & Wood (168) have proposed the following pathway to account for the conversion of pyruvate to propionate in *Propionibacterium shermanii*.



The first step involves the transcarboxylation of a one-carbon unit which is not in equilibrium with free CO_2 . The main pathway of propionic acid oxidation in animal tissues proceeds by the reaction sequence (11, 57, 172):

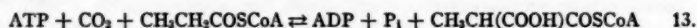


Ergerer *et al.* (51, 52), have reported that methyl malonyl isomerization to succinate takes place by migration of the thiol ester rather than the carboxyl group (12).

Much of the experimental activity in this area within the past year has been concerned with the role of vitamin B₁₂ and biotin in propionate metabolism. Smith & Monty (156) and Gurnani *et al.* (68) observed that the activity of methyl malonyl isomerase is severely depressed in the liver of vitamin B₁₂-deficient animals and that the activity could be completely restored (68) by the addition *in vitro* of 5,6-dimethyl benzimidazol-cobamide coenzyme, which is one of three cobamide coenzymes recently discovered, isolated, and characterized by Barker's group (8, 9, 183). Stimulation of methyl malonyl isomerase by this cofactor has also been reported in preparations from normal

animal tissues (163) and charcoal-inactivated extracts from propionic acid bacteria (52, 157). Lengyel *et al.* (107) have recently compared the relative activities of the three cobamide coenzymes in restoring activity to methyl malonyl isomerase apoenzyme prepared from sheep kidney and propionic acid bacteria.

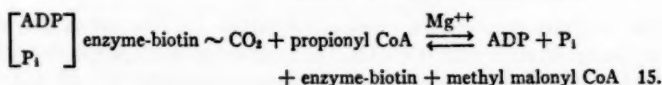
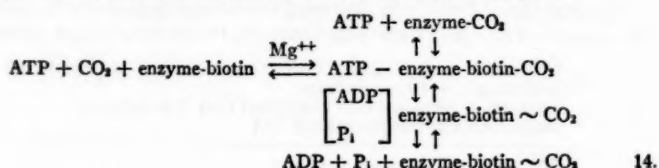
Propionyl carboxylase catalyzes the Mg^{++} -dependent reversible carboxylation of propionyl CoA to methyl malonyl CoA according to the following reaction:



The enzyme, which has been purified and characterized (71, 92, 172), is inhibited by avidin (71, 157).

Recently it has been reported that the highly purified pig heart preparation contains 10 moles of bound biotin per mole of enzyme (92). The fact that the biotin is not released by perchloric acid suggests that it is covalently bound. Covalent binding of biotin to enzyme has also been proposed in the case of the acetyl carboxylase of fatty acid synthesis (178).

Kaziro *et al.* (92), from isotopic exchange data have proposed the following mechanism for the reaction catalyzed by propionyl carboxylase.



They have been able to study the second step (Reaction 15) by preparing $C^{14}O_2$ -labeled protein (1 mole $C^{14}O_2$ per mole of biotin). When nucleotides are completely removed from the labeled enzyme, the $C^{14}O_2$ is not transferred to propionyl CoA until ATP is added. Mg^{++} is required for this step. Their experiments rule out the occurrence of steps involving the reaction of ATP with the enzyme to form an ADP enzyme or a phosphate-enzyme complex in the absence of CO_2 .

This mechanism therefore differs considerably from that proposed earlier by Lynen (113) to describe the sequence of reactions for the biotin-enzyme catalyzed carboxylation of β -methyl crotonyl CoA to β -methyl glutaconyl CoA.

A whole series of carboxylation reactions have been the subject of intensive investigation over the past year. Wakil has studied the biotin-enzyme-catalyzed carboxylation of acetyl CoA to malonyl CoA. This has been described in the section on fatty acid synthesis. Stern *et al.* (164) and Hegre

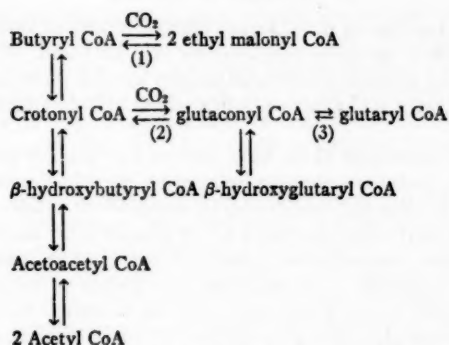


FIG. 3. Conversion of glutaryl CoA to β -hydroxyglutarate.

et al. (79) have investigated the carboxylation of butyryl CoA in mammalian enzyme systems and have identified the product of the reaction as 2-ethyl malonyl CoA (Figure 2, Reaction 1).

Testanoff & Stern (134) have recently demonstrated that crotonyl CoA is carboxylated to form glutaconyl CoA. These workers have discussed the possible role of glutaconyl CoA in the conversion of glutaryl CoA to β -hydroxyglutarate, recently described by Rothstein & Greenberg (143). They also present evidence suggesting that the conversion of glutaryl CoA to acetyl CoA proceeds by a pathway involving reversal of the carboxylation reaction. Figure 3 is the scheme they propose to summarize the various possible pathways.

HORMONAL REGULATION OF FATTY ACID METABOLISM

A number of hormones have been found to exert profound effects on fatty acid metabolism. In this section, only papers appearing during the past year have been cited; more comprehensive reviews of previous work in this area may be found in Langdon (103) and Fredrickson & Gordon (59).

Anterior pituitary hormones.—In general, anterior pituitary preparations appear to possess lipid-mobilizing activity (126); the major questions to which research has been directed concern the identity of the active hormones and the mechanisms by which their effects are exerted.

It has been reported that a crude alkaline extract of anterior pituitary, which causes hyperlipemia in rabbits, also causes the appearance in rabbit serum of an inhibitor of lipoprotein lipase (93) which was not present in the administered pituitary extract. Fractionation of such extracts has yielded a lipemia-producing material which is reported to be essentially free of the known trophic hormones (144). On the other hand, growth hormone preparations (63, 81, 103) and ACTH (114) have been found to cause prompt rises in serum free fatty acids, decreased triglyceride in adipose tissue stores (186),

and a delayed increase of other serum lipids (59) and of ketone bodies (81). Moreover, growth hormone preparations (91, 121) and ACTH (114, 121) have been found to cause accelerated oxygen uptake, increased net lipolysis, and decreased fatty acid synthesis when added to isolated rat adipose tissue *in vitro*.

Lynn and co-workers (114) have carried out balance studies aimed at elucidating the metabolic changes induced by the addition of ACTH to rat adipose tissue *in vitro*; the results may be summarized as follows. In the presence of ACTH and either glucose-1-C¹⁴ or glucose-6-C¹⁴ there was observed increased oxygen consumption, increased net glucose disappearance, increased C¹⁴O₂ production, increased net appearance of unlabeled free fatty acids and glycerol in the incubation medium, increased conversion of glucose-1-C¹⁴ to tissue glyceride-glycerol, and increased conversion of glucose-6-C¹⁴ to lactate. The conversion of glucose-6-C¹⁴, glucose-1-C¹⁴, or lactate-2-C¹⁴ to glyceride fatty acid or to glycogen was depressed, as was the conversion of added stearate-1-C¹⁴ to triglyceride. The lipolytic effect of ACTH was found to be independent of the presence of oxygen, substrate, or the occurrence of glycolysis. The authors suggest that ACTH stimulates lipolysis directly and that the other observed effects may result secondarily.

In contrast to the effects of ACTH and growth hormone, it has been reported (189) that prolactin, like insulin, stimulates the conversion to fatty acids of glucose-C¹⁴ or acetate-C¹⁴ by rat adipose tissue *in vitro*.

Posterior pituitary hormones.—Hashim (75) has recently reviewed evidence concerning the lipid-mobilizing hormone originally described by Seifter & Baeder (151). He has pointed out that administration of this hormone to either normal or hepatectomized animals results in hypertriglyceridemia; therefore, the view that the liver is necessarily the sole source of plasma triglycerides (33, 104) cannot be entirely correct. It is interesting that increased serum lipid-mobilizing activity has been detected (192) during pregnancy and may partially account for the hyperlipemia of gestation.

Another peptide (125) which results in lipid mobilization when administered to mice has been found in human urine and apparently is of hypothyseal origin. The relationship of this peptide to other pituitary hormones is presently unknown.

Epinephrine.—Epinephrine administration to intact animals results in a rapid rise in serum free fatty acid concentration (59, 154). This response is potentiated by adrenal steroid administration and does not occur in adrenalectomized animals (153). Sympathetic nerve endings in adipose tissue, which release norepinephrine, may physiologically exert an effect similar to epinephrine (22, 62, 76).

When added to adipose tissue *in vitro*, epinephrine causes net hydrolysis of tissue triglycerides leading to increased tissue free fatty acid concentration and increased free fatty acid release into the medium (105, 114, 121, 135, 161); accompanying this is released glycerol, which has its origin in pre-existing triglyceride (105, 114). Incorporation into glycerides of added long-

chain fatty acids is inhibited (114, 161), but the conversion of glucose-1-C¹⁴ to glyceride-glycerol is greatly accelerated (34, 114). Although net glucose utilization is enhanced (70, 114), its pathway of utilization is diverted from such normal products as fatty acids (114, 121) to the formation of lactate (70, 114) and CO₂ (34, 114); associated with increased CO₂ production is increased O₂ consumption (121). The R.Q. (corrected for lactate) remains relatively constant.

The lipolytic action of epinephrine in adipose tissue is independent of the presence of oxygen, substrates, or the occurrence of glycolysis (114) and is not secondary to activation of phosphorylase (161). Moreover, addition of free fatty acids to adipose tissue *in vitro* induces changes in glucose metabolism similar to those resulting from epinephrine. For these reasons it has been proposed (34, 114) that epinephrine in some primary manner accelerates the hydrolysis of stored triglycerides and that the other observed effects are secondary. Because epinephrine also inhibits the incorporation into triglycerides of added free fatty acids, it has been suggested (161) that net lipolysis results primarily from inhibition of triglyceride synthesis. Neither hypothesis has yet been unequivocally tested.

Glucagon.—The effects of glucagon on lipid metabolism in the intact animal (42, 54, 110) and adipose tissue *in vitro* (160, 121) resemble rather closely the actions of epinephrine. Therefore, they will not be enumerated here.

Thyroid hormones.—Past confusion concerning the effects of thyroid hormones on the rates of turnover of various lipids has resulted primarily from failure to ascertain the magnitude of the acetyl flux. Dayton *et al.* (45) have now reported experiments in which acetyl flux was measured; their results indicate that in the rat the absolute rates of synthesis of cholesterol and fatty acid are directly proportional to the basal oxygen consumption. This may be correlated with the observation that stimulation of fatty acid synthesis by insulin is more pronounced and more sensitive than normal in adipose tissue from hyperthyroid rats (69). It may also be relevant that the enzymes of the hexose monophosphate pathway are decreased in the tissues of hypothyroid rats (89). In keeping with the well-known calorogenic action of thyroid hormones on cardiac musculature, it has been found (47) that butyrate oxidation proceeds at accelerated and decreased rates in homogenized heart muscle from hyper- and hypothyroid rats respectively.

Diabetes and insulin.—The characteristic elevation of serum free fatty acids observed in the venous blood of diabetic humans (59, 120) and lower animals has generally (59, 184) been attributed to an increased rate of release of those acids from adipose tissue into the bloodstream. Conversely, the decrease in serum free fatty acids following insulin injection has been considered to result from a diminished rate of entry of free fatty acids without alteration in their rate of utilization (48, 53, 155). This view is consistent with data demonstrating that net release of free fatty acids by adipose tissue *in vitro* is accelerated in diabetic tissue (184) and that insulin plus glucose is effective in suppressing fatty acid release from normal adipose tissue *in vitro*

(128, 191). However, Butterfield & Schless (32) find that the concentration of free fatty acids is not greatly different in the arterial plasmas of normal and diabetic subjects but that the net rate of uptake by the muscular forearm is diminished from the normal value of 3.6 m.eq./100 ml. forearm/min. to only 0.17 m.eq. in diabetic man. The normal rate is restored by insulin treatment. Inasmuch as insulin in the presence of glucose also promotes uptake and esterification of added isotopically labeled fatty acids by adipose tissue *in vitro* (7, 114, 128), it seems reasonable to suppose that suppression of release and enhancement of uptake both are involved in regulating serum free fatty acid concentrations by insulin; indeed, they may both simply reflect increased fatty acid esterification resulting from increased availability of α -glycerol phosphate (161).

The rate of release of free fatty acids from adipose tissue appears to depend upon the intracellular concentration of unesterified acids, which in turn is the result of a balance between the rates of triglyceride synthesis and hydrolysis. Recent data (7, 114) suggest that the effect of insulin on this balance is indirectly mediated by increasing the rate of glucose entry and hence the availability of α -glycerol phosphate for triglyceride synthesis.

Chaikoff and his co-workers (1, 2, 117, 118) have reinvestigated the metabolic lesion responsible for depressed fatty acid synthesis (58, 190) in the tissues of diabetic animals; they have obtained evidence which implies that, contrary to previous suggestions (103), defective lipogenesis in diabetic liver cannot be attributed to a diminished rate of NADP reduction. Instead, they report that the activity of NADP-ethylene reductase (103) is decreased to only 20 to 30 per cent of its normal value in microsomes from diabetic rat liver, and they suggest that this enzymic alteration may be partially responsible for depressed lipogenesis in diabetic liver. It has also been reported (72) that oxidative phosphorylation is partially uncoupled in hepatic mitochondria from alloxan-diabetic or pancreatectomized rats, and the possibility has been entertained that the consequently diminished availability of ATP for synthetic reactions may be of importance. Actually, the concentration of adenosine monophosphate is increased in diabetic liver, whereas both ATP and ADP are decreased (19); the ratio of the latter two nucleotides is not abnormal.

The rate of oxidation of palmitate-1- C^{14} to CO_2 has been reported (56) to be increased twofold in diaphragms from alloxan-diabetic or fasted rats; however, glucose addition spared palmitate oxidation by diabetic or normal fasted diaphragms and by adipose tissue (7). Although insulin enhanced this sparing action of glucose in adipose tissue (7), the hormone had no effect on palmitate oxidation by resting or stimulated rat diaphragm (55).

The significance for lipid metabolism of the increased pinocytotic activity of adipose tissue in the presence of insulin (10) has not been assessed.

CHOLESTEROL ABSORPTION

It is well-known that both dietary and endogenous unesterified cholesterol are absorbed from the gastrointestinal tracts of laboratory animals and

transported into the lacteals; during this process, cholesterol is esterified by a process which is not yet understood. Hellman *et al.* (80) have extended these studies to human subjects with cannulated thoracic ducts and have found that dietary cholesterol is recovered primarily in esterified form from thoracic duct lymph. Absorption and esterification apparently occur in the upper jejunum (23), which is also the site of triglyceride absorption.

Apparently the esterification reaction is in some way essential for cholesterol absorption. Swell *et al.* (165) have obtained data which suggest that, prior to absorption, dietary cholesterol oleate is hydrolyzed and that only free cholesterol can enter mucosal cells. Although dihydrocholesterol is also esterified during absorption (174), esterification cannot be generally required for steroid absorption, since the methyl ether of cholesterol (64), as well as cholestenone and epicholesterol, is absorbed as such.

Comparison of the fatty acid components of the cholesterol ester of rat thoracic duct lymph during fasting and after ingestion of a meal consisting of cholesterol and oleic acid has revealed (167) that, although palmitic and linoleic acids predominate in fasting lymph, oleic acid becomes the principle component after oleic acid ingestion. However, 58 per cent of the esterified acids still arise from endogenous sources.

It is clear (18, 136, 166) that the fatty acid composition of the cholesterol esters in serum lipoproteins bears little resemblance to the dietary fatty acids. Part, although certainly not all, of this difference may be attributable to the initial absorption process.

Inquiry into structural specificity for sterol absorption has revealed that cholesterol methyl ether (64), dihydrocholesterol (174), coprostanol (6), β -sitosterol (147, 185), and sitosterol acetate (147) are absorbed from the gastrointestinal tract, although, in general, cholesterol is absorbed more efficiently than the other steroids.

STEROID DEGRADATION

Bergstrom and his colleagues have continued their work on the metabolism of cholesterol to bile acids and other end products. This work has been well-summarized in a recent review (17). It has become evident from studies in different species that those bile acids which are products of hepatic synthesis have 7 α -hydroxyl functions. 7-deoxy acids, which occur in bile, result from dehydroxylation of the primary acids by intestinal microorganisms and recirculation of these acids with or without further hepatic modification. Deoxycholic (145), hyodeoxycholic (16), and pythocholic acids (15) have all been found to result from this pathway. 6- α -Hydroxylation of chenodeoxycholic acid (16), resulting in hyocholic acid, and 6- β -hydroxylation of deoxycholic acid (131) to yield 3- α -, 6- β -, 12- α -trihydroxycholanic acid have been reported to occur in the pig and rat respectively; both of these hydroxylations apparently occur in the liver.

Danielsson (40) has made the very interesting observation that mouse or rat liver mitochondria convert 3- α -, 7- α -, 12- α -trihydroxycoprostanol to 3- α -, 7- α -, 12- α -, 27-tetrahydroxycoprostanol. The latter compound, when

incubated with hepatic mitochondria plus the high speed supernatant fraction of rat liver, was converted to 3- α -, 7- α -, 12- α -trihydroxycoprostanic acid, a compound that had previously been found to be a precursor of cholic acid in intact rats. Whitehouse *et al.* (187) have reported that incubation of cholesterol-26-C¹⁴ with rat liver mitochondria results in the production of acetone-methyl-C¹⁴, whereas, under the same circumstances, acetone-2-C¹⁴ is produced from cholesterol-25-C¹⁴. Since acetoacetate-1-C¹⁴ was not detected in either case, these authors conclude that ω -oxidation of the cholesterol side-chain followed by oxidation to a β -keto acid and removal of two terminal carbons as acetyl CoA is improbable.

Although, in the past, it has been assumed that the primary endogenous source of neutral fecal sterols, such as coprostanol, is biliary cholesterol, it has been found (41) that neutral fecal sterol excretion is actually increased twofold in bile fistula rats. Since intraperitoneally administered cholesterol-4-C¹⁴ was converted to fecal sterols, it is apparent that account must be taken of cholesterol excretion by the intestinal mucosa. A strain of bacteria that converts cholesterol to coprostanol without the addition of cerebroside has been obtained from human feces (127).

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CARBOHYDRATE METABOLISM^{1,2}

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The increasing complexity of the once simple field of carbohydrate metabolism makes a selection of topics imperative for a useful critical approach within a limited space. Current trends and personal experience have contributed to the selection of the following subjects: the transport of sugars, glycolytic enzymes, and certain complementary and alternative pathways.

TRANSPORT OF SUGARS

There is an increasing awareness that the passage through cell membranes is a critical step for many metabolites. The sugars, major metabolic fuels for most cells, are now second to none in the attention currently paid to the transport problems. Interest has been increasing in the last decade, particularly during the last few years. The realization that the membranes are not sievelike bags but barriers to a large extent impermeable to glucose makes it obvious that some sort of mechanism is likely to intervene to enable an adequate supply of glucose to reach the intracellular glycolytic machinery.

Ever since Levine pointed out some 10 years ago the permeability problem in the penetration of sugars in animal tissues, a large proportion of the work on transport of sugars has been aimed at the examination of its likely involvement in the mechanism of action of insulin. This particular interest has been a great stimulus but also a hindrance. Too much was attempted too soon. Tissues known to be, or likely to be, targets for insulin action have been used preferentially for experimental work on transport, despite the fact that most of them are highly undesirable as experimental tools for the investigation of mechanisms of transport across cell membranes. Thus, there has been a piling up of frequently conflicting results. Transport for the sake of transport, i.e., as a basic problem of cellular physiology, has been independently studied in simple experimental systems, such as cell suspensions, mostly of microorganisms, and in intestinal mucosa, quite unrelated to the "insulin-stimulated" work.

Stereochemical specificity has been in most cases the major factor to rule out simple diffusion in the uptake by cells or tissues or transport across them. In contrast to this fact, it appears now, as a result of recent, more detailed investigations, that the structural requirements for transport seem to be too

¹ The survey of the literature pertaining to this review was concluded in August, 1960.

² The following abbreviations are used: DNP for dinitrophenol; NAD for nicotinamide-adenine dinucleotide; NADH₂ for nicotinamide-adenine dinucleotide, reduced form; NADP for nicotinamide-adenine dinucleotide phosphate; UDP for uridine diphosphate.

vague for clear definition. They do not seem to agree with common experience on the substrate specificity of enzymes. LeFevre & Marshall (1) have suggested that the over-all geometrical shape of the sugar molecule could be a major factor. Another possible general explanation can be drawn from the fact that transport systems, although they have kinetics similar to those of enzymes, do not seem to give a modified substrate as a product. The likelihood of a three-point attachment, pointed out by Ogston and emphasized for enzymes acting on sugars by Gottschalk, stems from the obvious need for attachment of the substrate to the enzyme in a definite spatial orientation for the action of the enzyme to be possible. If three points were also involved in the attachment to a carrier of its main substrate, it is conceivable that the loss of any one of them could be compatible with a significant residual complexing capability, presumably the only essential requirement for "activity" in the case of transport. After all, the seemingly wide specificity pattern of transport systems is not so different from those frequently found with enzymes if both substrates and competitive inhibitors are taken into account. Moreover, it must be emphasized that precise quantitative evaluation is more difficult, and certainly has been less developed, for transport systems than for some enzymes. Dependence on qualitative or semiquantitative data can be considerably misleading in this respect. In addition, there is the possibility that in a particular tissue two or more transport systems for sugars with different specificities might be involved.

Weiss (2) has formulated the hypothesis that specific molecular interactions at the cell surface may unlock less specific and dynamically more potent transport and transmission mechanisms.

There is no agreement in nomenclature. Terms like permeases, transportases, or translocases are certainly descriptive but presently seem to imply too much analogy with ordinary enzymes. The more general designation of transport systems will be preferentially used, except in bacteria where permeases have been generally accepted.

Mitchell (see 3) supports the theory that transport occurs as a consequence of anisotropic arrangement of enzymes relative to structures such as membranes. This attractive idea is encouraged by the observation that a number of enzymes form part of certain membranes. Although there are already a number of well-authenticated cases of transport systems independent of metabolic utilization, this theory may apply in yet others.

Escherichia coli.—The brilliant work of Monod and co-workers, which started with the identification in 1956 of a galactoside permease in *E. coli* (see 4), has led to the characterization of several other permeases for sugars and to kinetic studies which might contain clues to the mechanisms involved. Labelled β -thiogalactosides were found to be accumulated by adapted cells. Since apparently they were not metabolized, the accumulation indicated an intracellular concentration many times that of the medium. Independently, Pardee (5) had observed an accumulation of the α -galactoside melibiose which seems to be mediated by the same galactoside permease. Siström (6)

has shown that osmotically active spheres prepared by lysozyme treatment respond osmotically to the addition of galactosides, which indicates that the bulk of the accumulated galactoside is an intracellular free solute. Light scattering may also afford a direct assay of transport phenomena in intact *E. coli* according to Packer & Perry (7). An acetyl coenzyme A-dependent acetylation which has been suspected to be carried out by a system closely connected with, or part of, the permease system has been identified (8). On the other hand, this identification of a metabolic transformation of a thiogalactoside could be related to the apparent concentration against a gradient. Accumulation per se, even if osmotically active, does not permit an unreserved estimate of intracellular concentration of an unchanged substrate of a transport system. A cryptic strain slowly hydrolyzed *o*-nitrophenyl- β -galactoside with first-order kinetics throughout the range of concentrations tested, confirming the absence of galactoside permease [Herzenberg (9)] or a drastic decrease in affinity for the substrate. The galactoside permease-less, β -galactosidase constitutive mutant has been utilized by Brock (10) to measure changes in impermeability by observing the efficiency to hydrolyze *o*-nitrophenyl- β -galactoside.

As for mechanism, Monod's school supports a carrier theory in which, as developed by Kepes (11), accumulation of a non-metabolizable substrate by the cell is considered to represent a balance between an active entry process, which seems to involve one mole of ATP per mole transported, and an exit mechanism, which depends on the concentration gradient but must be considered as a carrier diffusion.

Rotman (12) reported the perplexing observation that a galactosidase-less mutant could accumulate methyl- β -galactoside constitutively, whereas an accumulation of methyl- β -thiogalactoside occurred only after previous induction. The conclusion that a new permease was involved in the uptake of the methyl- β -galactoside has been substantiated by Osborne, who found the latter to be a marginal substrate of a galactose permease (see 13). The transport of galactose has been studied by Horecker, Thomas & Monod (13, 14) in a galactokinase-less mutant. This organism can accumulate galactose to levels that greatly exceed those in the external medium. The stereospecific transport system has a high affinity for galactose (K_m ca. 10^{-5} M) and is sensitive to DNP. The observation that the initial rate of galactose uptake is identical in the wild type and in the mutant strain, whereas in the former no free galactose accumulates, suggests that permeation is the limiting step in galactose utilization. The steady state level of galactose accumulation in the galactokinase-less mutant is interpreted as the result of a balance between rate of uptake and rate of exit. Apparently the latter can be increased or decreased without corresponding changes in the activity of the entry system. The results are generally in agreement with Kepes' transporter model, except for a stimulating effect of either low concentrations of glucose or of succinate on the accumulation level. Further work by Horecker *et al.* (15) on the effect of DNP on transport and accumulation indicates that it is not

the entry that is affected but rather an additional mechanism involved in the accumulation. From observations in a mutant that prefers galactose to glucose, Horecker *et al.* (15) have obtained indirect evidence indicating that the basis for normal diauxie might be at the transport level. Evidence for a constitutive galactose permease had also been obtained by Glass & Novick (16).

A maltose permease has been identified by Wiesmeyer & Cohn (17). An amylomaltase-less mutant can, after induction, accumulate ^{14}C -labelled maltose. The accumulated carbohydrate is largely maltose, although other compounds have been also detected. It cannot be removed by washing, but it exchanges in part with externally added maltose. Accumulation is prevented by sodium azide. This permease is different from the galactoside, galactose, and glucose permeases. The last has been previously detected (4) but has not yet been characterized (see also 18). There are indications that the synthesis of the maltose permease may be normally restrained by an inhibitor that can be suppressed by x-radiations [Laser & Thornley (19)]. Ribose utilization seems also to involve an inducible permease [Eggleston & Krebs (20)].

Yeasts.—Some yeasts are outstanding in their very high rate of fermentation of certain sugars. In contrast with this fact, Conway showed that bakers' yeast is highly impermeable to monosaccharides. On the other hand, Rothstein showed that non-penetrating inhibitors could interfere with the utilization of the fermentable hexoses. Sols identified a constitutive stereospecific step which occurs prior to the phosphorylation involved in the uptake of glucose, fructose, and mannose (21). Another transport system is involved in the uptake of galactose [Sols, de la Fuente & Alvarado (22); de Robichon-Szulmajster (see 23)].

Both the basic impermeability and the occurrence of stereospecific transport systems have been confirmed by the finding of Burger, Hejmová & Kleinzeller (24) and Cirillo (25) that certain non-fermentable monosaccharides are selectively transported up to an apparent intracellular concentration which approaches equilibration with the medium. According to Cirillo's observations, the kinetics correspond to a very small affinity, which is consistent with the hypothesis that their transport is a marginal activity of a system normally involved in the transport of fermentable sugars. According to Kotyk (26), even glucose can be detected in an apparently free form in certain conditions, particularly one to two minutes after the addition of glucose to a suspension of yeast under aerobic conditions. 2-Deoxyglucose is taken up by yeast and accumulates as the 6-phosphate. The rate of uptake parallels that of glucose [Kiesow (27)]. 2-Deoxygalactose is taken up by galactose adapted yeast [Heredia, de la Fuente & Sols (28)].

Some oligosaccharides are also very readily utilized by certain yeasts. Robertson & Halvorson (29) found evidence for an inducible labile "permease-like" factor involved in the utilization of maltose. Sols & de la Fuente (30) have shown that stereospecific transport systems are the first step in

the utilization of maltose and certain other α -glucosides by *Saccharomyces cerevisiae* and lactose and certain other β -galactosides by *Saccharomyces fragilis*, whereas the normal pathway of utilization of sucrose and β -fructosides and melibiose and α -galactosides involves extracellular hydrolysis by ectoenzymes located in the periphery of the cell wall, followed by transport of the liberated hexoses. The old question of direct versus indirect fermentation of oligosaccharides resolves itself into transport before or after the corresponding glycosidase. Removal of the cell wall in the preparation of protoplasts is accompanied by loss of invertase [Friis & Ottolenghi (31); Sutton, Marini & Lampen (32)] or melibiase (33). For isomaltose and certain other α -glucosides there is evidence that a transport system different from that of maltose as well as a different glucosidase is involved [Terui, Okada & Oshima (34); (24)]. Indication that complementary mechanisms might be involved in certain yeasts is apparent from the observations of sucrose and trehalose accumulation in haploid yeast hybrids [Avigad (35)].

The transport systems for sugars in yeasts are particularly important since the combined evidence available strongly suggests that transport is the limiting step in the utilization of glucose and other readily fermentable sugars.

Other microorganisms.—Little work on transport of sugars has been carried out in microorganisms other than *E. coli* and yeasts. Nevertheless, since the permeases are now highly fashionable, there is a tendency to assume the occurrence of specific permeases on the slightest evidence or even without any real evidence at all. Such treatment makes it difficult to generalize. In fact, the reviewer feels that a well-conducted study of cases in which possibly no sugar transport system at all is involved would be more useful. It must be realized that not even *E. coli* and the *Saccharomyces* are entirely impermeable to sugars; the well-authenticated transport systems in these cases make possible rates of uptake many times greater than those that would be possible by mere diffusion, especially at low concentrations. On the other hand, some other microorganisms may have membranes with a smaller degree of impermeability to sugars, and certainly many microorganisms have metabolic rates which do not require any very fast penetration of carbohydrate substrate.

A very peculiar permeability phenomenon has been observed by Abrams (36, 37, 38). The membrane of *Streptococcus faecalis*, virtually impermeable to oligosaccharides and several non-related compounds (but not to monosaccharides), appears to change during glycolysis to permit penetration at a highly enhanced rate. This conclusion stems from the observation that protoplasts osmotically stabilized in a sucrose solution undergo a metabolic swelling upon the addition of glucose in the presence of K^+ . The swelling slowly reverses upon exhaustion of the glucose added. ^{14}C -labelled sucrose is taken up during the swelling. ^{14}C -labelled substances leave the protoplasts during the reversal, presumably in the form of products of sucrose metabolism. Phlorizin enhances the uptake of the "impermeant" sugar. Abrams relates this phlorizin effect to the long-known effects on transport

across other membranes, although here the effect is of opposite sign. This apparently unspecific penetration provoked by glucose would be a case of the specific unlocking of relatively unspecific transport mechanisms proposed by Weiss (2). Abrams has further observed that intact cells suspended in 0.4 *M* sucrose undergo lysis if glucose is added. The same occurs with *E. coli* [see also Packer & Perry (7)]. No explanation is apparent.

Erythrocytes.—As isolated cells of animal origin, erythrocytes have long been a favorite experimental subject, or rather subjects, because here species differences are so great that attention to the origin is essential and not to be forgotten when comparing results.

The subject has been recently reviewed by Wilbrandt (39) and by Park *et al.* (40). The accumulated evidence establishes the existence in human erythrocytes of a mechanism that facilitates the passage through the membrane of certain monosaccharides, with kinetics strongly supporting a carrier system. Especially significant is the observation of a flow-induced uphill transport by Park and co-workers and by Rosenberg & Wilbrandt (41). Lacko *et al.* (42) have observed that this exchange of sugars between medium and cells is at 0° much faster than the uptake by sugar-free human erythrocytes. LeFevre & Marshall (1) have observed that the apparent affinities of different hexoses and pentoses roughly parallel the sequence of increasing relative stability in the particular chair shape designated as the C1 conformation.

In addition to the well-known strong inhibitory effect of phloretin on sugar transport in human erythrocytes, LeFevre (43, 44) has found that some other diphenolic compounds are also strong reversible inhibitors. In fact, diethylstilbestrol is even more strongly inhibitory than phloretin. The erythrocytes bind these compounds rapidly and reversibly. The relative efficiency of binding seems to parallel their inhibitory power on sugar transport, but the fact that the binding proceeds to the same distribution ratio at all concentrations tested clearly rules out the exclusive involvement of the presumed sugar transport sites.

The slow penetration of glucose in rabbit erythrocytes has no apparent structural requirements. Several alkyl derivatives of glucose have been found to penetrate faster than glucose, which suggests that in this case lipid solubility may be a major factor [Hillman, Landau & Ashmore (45)].

Recent observations by Wilbrandt (39) suggest that more than one transport system for monosaccharides might exist in human erythrocytes. Two systems with partially overlapping specificity patterns would make it particularly difficult to obtain a clear picture of the specificity requirements.

Ascites tumor cells.—In contrast with the erythrocytes, ascites tumor cells, while also being individual cell suspensions, have a very high rate of glycolysis. Accordingly, the mechanism of entrance of sugars is of particular interest. Crane, Field & Cori (46) reached the conclusion that the penetration of hexoses and pentoses into the Ehrlich ascites tumor cells can be described as a reversible first-order process with an equilibrium ratio of in-

side and outside concentrations of one. The rate of penetration of a series of sugars indicates that the cell membrane has configurational specificity. Penetration does not appear to be rate-limiting for glucose but can be so for fructose. These conclusions are supported by the independent observations of Nirenberg & Hogg (47) on glycolysis and fructolysis and the effects of galactose upon them in Ehrlich carcinoma and Gardner lymphosarcoma ascites tumor cells. Yushok (48), working with Krebs-2 ascites carcinoma, concludes that the competition between the glycolyzable hexoses can be partly explained by the relative efficiencies of its hexokinase, although the lack of effect of several inhibitors of hexokinase on intact cell glycolysis is compatible with the involvement of a specific step prior to phosphorylation (49).

Intestinal absorption.—The substrate specificity approach, which first showed that the active absorption of sugars from the intestine does not involve their phosphorylation [Sols (50)], has been greatly developed by Crane, Wilson, and collaborators. The process, for a long time believed to be narrowly selective, has been drastically changed to include a large variety of sugars and sugar derivatives that can be actively absorbed by the isolated surviving hamster intestine (51, 52). Every hydroxyl group in the glucose molecule is dispensable without loss of capability for active absorption, except the one at carbon 2. 2-Deoxyglucose is not absorbed nor does it inhibit glucose absorption [Sols (53)]. The minimal requirements are only a D-pyranose structure, a methyl or substituted methyl group at carbon 5, and a hydroxyl group in the glucose configuration at carbon 2. Nevertheless, competition experiments are consistent with the view that all transported sugars compete for a common pathway (54, 55). In a deeper analysis of the likely involvement of the substituents at carbon 2, Crane & Krane (56) have carried out studies with $H_2^{18}O$, glucose-2- ^{18}O , and 2-C-hydroxymethylglucose with results that exclude as intermediate steps of active transport those reactions that involve the removal or transfer of oxygen at carbon 2 or require the presence of carbon-bound hydrogen at the same position.

The well-known partial transformation of fructose into glucose in the intestine has been now clarified. The transformation is considerable in guinea pig and hamster but not in the rat. Using fructose and glucose labelled with ^{14}C in a single carbon atom, Salomon & Johnson (57) and Ginsburg & Hers (58) have obtained evidence that the fructose to glucose conversion in the guinea pig intestine involves chain fragmentation. Ginsburg & Hers have further identified the occurrence of fructokinase and glucose-6-phosphatase activities in the intestinal mucosa of the guinea pig; the latter activity is not detectable in the rat. Two different enzymes with ketokinase activity have been identified by Cadenas & Sols (59) in the intestinal mucosa of several species of mammals. Moreover, attempts to detect direct isomerization by homogenates have been consistently negative (58, 59). These observations lead to the conclusion that the conversion of fructose to glucose by the intestinal mucosa of the guinea pig occurs by the same mechanism as operates

in the liver; fructose-1-phosphate and triose phosphates are involved as intermediates. It is still an open question whether this transformation has any direct relationship to the removal of fructose from the intestinal lumen somewhat faster than the pentoses; alternatively, it could be activity complementary to that known to occur in the liver of several species.

It has been recently established that the cations present in the medium can greatly influence the absorption of sugars by isolated surviving intestine. Ricklis & Quastel (60, 61) found with guinea pig intestine that Na^+ was essential and that K^+ (or Rb^+) could considerably increase the rate of absorption at low glucose concentrations. Their results support the conclusion that K^+ affects a specific phase involved in the active transport of sugars rather than a change in permeability. DNP and phlorizin inhibit the potassium-stimulated transport at lower concentrations than those required to inhibit the unstimulated transport. The Na^+ requirement has also been demonstrated for the toad [Csáky & Thale (62)], rat [Csáky & Zollicoffer (63); Parsons & Wingate (64)], and hamster intestine [Crane *et al.* (55)], and it has been suggested that sugar transport might be linked with Na^+ transport.

Smyth and co-workers (65, 66) have shown that phlorizin can inhibit the transport of glucose by isolated intestine at concentrations that do not affect endogenous metabolism. The results suggest that phlorizin acts on the mechanism responsible for the passage of glucose across the membrane in the luminal side of the mucosal cell. It inhibits also the absorption of fructose, but to a lesser extent [Giráldez & Larralde (67)].

According to Ponz & Lluch (68), the intestinal absorption of glucose in the rat can be increased by intravenous administration of cytochrome-*c*. And according to Nissim (69) the bromide ester of trimethylhexadecylammonium, a surface-active agent, inhibits glucose absorption in the rabbit at concentrations that do not affect methionine or butyrate absorption nor seem high enough to cause structural damage.

It has been assumed that disaccharides are not actively absorbed but undergo preliminary hydrolysis by secreted enzymes or ectoenzymes. The intestinal mucosa has several α -glucosidases, one of which splits sucrose [Dahlqvist (70)]; and in suckling mammals there is also a β -galactosidase which splits lactose (71). Human cases of congenital inability to utilize lactose [Holzel *et al.* (72)] or sucrose [Weijers *et al.* (73)], apparently by lack of the corresponding glycosidase, have recently been identified. However, there is still the possibility that disaccharides are in part transported as such and afterwards split intracellularly.

Kidney.—The second major organ of sugar transport, the kidney, has been much less studied than the intestine, largely because of technical difficulties. Krane & Crane (74) have tested a novel approach, which was borrowed from the techniques recently developed for studies of uptake by cell suspensions. Galactose-1- ^{14}C is accumulated by slices of rabbit kidney cortex against an apparent concentration gradient in apparently unmodified form. Accumulation does not occur in anaerobiosis or in the presence of dinitro-

cresol. Phlorizin inhibits the entrance of galactose into the tissue. Wick & Serif (75) have observed that 6-deoxy-6-fluoroglucose inhibits the utilization of glucose by kidney slices in a way that points to interference with a transport process. Meanwhile, Chinard *et al.* (76) have shown that renal reabsorption in vivo, in the dog of glucose-1-¹⁴C or glucose-6-¹⁴C is not accompanied by randomization, which indicates that glucose is transported across the renal tubule cells with the 6-carbon chain intact.

Placenta.—The placenta is an organ of transport between mother and foetus. It is known that foetal blood contains large amounts of fructose in ungulates and smaller amounts in other mammals. As indicated by permeability studies, a stereospecific transport of glucose seems to be involved.

According to Walker (77), glucose, mannose, galactose, and xylose in the goat can pass rapidly across the placenta in both directions, whereas it appears to be impermeable to fructose. He postulates a transport process specific for aldoses and suggests that the impermeability to fructose might be a general phenomenon unrelated to fructose formation but helping to maintain high concentrations of fructose in the cases in which it is formed. This conclusion is supported by the observations of Karvonen & Leppänen in sheep (78). Where, how, and for what purpose the foetal fructose is formed is still an open problem. Based on the discovery that sheep placenta contains a reductase which can convert glucose to sorbitol and on the well-known occurrence in liver of a sorbitol dehydrogenase, Hers (79) postulates that foetal fructose might be formed in the foetal liver out of sorbitol formed in the placenta. However, Hagerman, Roux & Villee (80) have found ketose reductase but not aldose reductase in human placenta, which *in vitro* forms both fructose and glucose, as could be expected from the combined action of an unspecific phosphomonoesterase and phosphoglucose isomerase. In apparent contrast with the high fructose content of foetal blood, Hers has found that the liver fructokinase is absent in the foetus (see also 79a). In fact, the absence of liver fructokinase plus the impermeability of the placenta could explain the accumulation of fructose in blood. If there is any direct physiological role for foetal fructose, it must be in the carbohydrate metabolism of peripheral tissues.

Muscle.—Work directly or indirectly related to the permeability and transport of sugars in skeletal and heart muscle is part of many recent and current studies on the action of insulin. Various aspects of this subject have been recently reviewed by several workers in this field: Park and collaborators (40, 81), Kipnis (82), Goldstein (83), Fisher (84), Randle & Young (85), and Randle (86). There is increasing support for Park's original contention that in muscle the membrane transport of glucose is the limiting step in the absence of insulin and that the transport is activated by insulin so that the limiting step shifts from transport to phosphorylation. Glucose transport can also be activated by some humoral factor that is released directly from strenuously contracting muscle (83).

Fisher & Zacharias (87) have observed that the kinetics of penetration

of L-arabinose and D-xylose into the perfused rat heart conform to the carrier mechanism proposed by Widdas for the human erythrocyte. In the absence of insulin, the half-saturation concentrations are *ca.* 10^{-4} M; in its presence, they are *ca.* 10^{-2} M. Thus, insulin appears to reduce the affinity of the carrier for sugar. It also reduces the constant corresponding to the amount of carrier involved in the permeation process (to about one-third in the presence of 0.2 units/l). These changes, though at first glance in the wrong direction, can account for the effect of insulin in increasing permeation. The affinity of the carrier for the sugars in the absence of insulin is so high that when only a little sugar has penetrated into the cells the degree of saturation of the carrier is nearly the same on both sides of the membrane and the net penetration rate becomes infinitesimal. The formation of an insulin-carrier complex with a lowered affinity for the substrate would then increase the actual efficiency of the system; and the more efficient insulin-carrier complex would rapidly result in attainment of an internal sugar concentration sufficiently high to make the unmodified carrier ineffective, i.e., the penetration observed in the presence of insulin would be almost entirely caused by the fraction of the carrier combined with insulin.

In vitro preparations, particularly the cut diaphragm, involve too great a danger of artifacts to be used in permeability studies. The insulin-non-responsive Embden-Meyerhof system observed by Shaw & Stadie (88) is likely to correspond to cut fibers and escaped enzymes trapped in the interstitial spaces. Another danger lies in the possibility of decrease in "impermeability" in certain conditions. Heart muscle seems to become permeable to sucrose if nitrate is substituted for the chloride in the medium [Taketa *et al.* (89)].

Walaas *et al.* (90) have observed a rapid incorporation of ^{14}C -glucose into a glucan-peptide complex of the diaphragm; the incorporation is accelerated by insulin and could possibly be related to the carrier in the transport of glucose.

Fructose has been repeatedly observed to be apparently inert for the glucose transport system. An observation of Pearson & Rimer (91) in a case of McArdle myopathy suggests that, in humans, fructose can traverse the muscle cell membrane more readily than glucose at low plasma concentrations.

Adipose tissue.—This tissue, until recently considered an inert storehouse, is now the subject of intensive study as a major target of insulin. The rate of hormone-stimulated glucose uptake, in terms of tissue protein, is remarkably high [Jungas & Ball (92)]. Nevertheless, there is no direct evidence of whether transport of glucose occurs as a first step [Jeanrenaud & Renold (93)]. That it is likely to be the first step is suggested by the marked differences in the available data on the relative efficiencies of the epididymal fat pad of the rat *in vivo* and *in vitro* with glucose, mannose, and fructose. These sugars are metabolized by the intact tissue at relative rates of *ca.* 1.0, 1.0, and 0.2 [Cahill *et al.* (94)], and their relative responses to insulin

are 1.0, 0.66, and 0.34, respectively [Ball & Cooper (95)]. On the other hand, this tissue has an hexokinase whose relative maximal phosphorylation rates and Michaelis constants for the three sugars are, respectively, 1.0, 1.6, and 1.3 and 3×10^{-3} M, 5×10^{-3} M, and 3×10^{-3} M [Hernández & Sols (96)]. The activity of the various enzymes acting on glucose-6-phosphate have been studied by Weber *et al.* (97).

Miscellaneous animal tissues.—Leucocytes have high glycolytic rates and as cell suspensions could be an important experimental tissue, although it is not clear whether their uptake of glucose is stimulated by insulin. Luzzatto (98) reports that xylose enters human leucocytes tending to equilibration and that the rate of entrance is increased by insulin.

Lymph node cells prepared in the form of single cell suspensions have been studied by Helmreich & Eisen (99) with respect to permeability to glucose and other sugars. The results are thought to indicate that glucose transport across the cell membrane is not limiting, although access to some intracellular sites can be limiting. The sugars tested compete for penetration. Phlorizin and intracellularly accumulated 2-deoxyglucose-6-phosphate inhibit non-competitively.

Rickenberg & Maio (100) have applied the techniques developed for the study of sugar transport in *E. coli* to an examination of sugar transport by mammalian cells grown in tissue culture. The L-cell strain of fibroblasts, grown as a suspension of individual cells, seems to accumulate galactose against a concentration gradient. Nevertheless, the apparently great concentration factor has been found to depend, at least to a large extent, on metabolic transformation of the apparently inert galactose. This realization instills an element of caution into the over-all importance that tends to be given to any "demonstration" of uphill transport. On the other hand, Agol (101) reports that, in several mammalian cell cultures, galactose rapidly penetrates in the intracellular water up to an apparent concentration slightly smaller than that in the medium. The difference from the above observations probably does not depend on the origin of the cultures but simply on the fact that Agol used a more conventional approach involving the use of concentrations of galactose in the medium (*ca.* 0.007 M) much greater than those usual in the work with labelled compounds.

GLYCOLYSIS

Glycogen metabolism.—Of outstanding importance are the recent developments in the uridine diphosphate glucose-linked mechanism as the major physiological pathway of glycogen synthesis.

Villar-Palasi & Lerner have purified the UDP-glucose pyrophosphorylase from skeletal muscle. The enzyme seems to be specific for α -glucose-1-phosphate, and has high apparent affinity for UDP-glucose and magnesium pyrophosphate (102). They have also studied the distribution of the pyrophosphorylase, phosphorylase, and phosphoglucomutase in rat tissues (103). Their results, together with those of Leloir *et al.* (104) on the distribution of

the glycogen synthetase, clearly indicate the general validity of the cycle shown in Figure 1.

Reaction 2 of Figure 1 appears to be virtually irreversible. And Reaction 3, although clearly reversible *in vitro*, seems to work only in the phosphorytic degradation of glycogen under the range of conditions known to prevail *in vivo*. Further support for the actual validity of the cycle (Figure 1) stems from the biochemical identification of the defect in a muscle disorder characterized by a rapid exhaustion which can be prevented by the intravenous administration of glucose. The studies of Mommaerts *et al.* (105), Schmid, Robbins & Traut (106), and Lerner & Villar-Palasi (107) clearly indicate that the enzymatic basis is the virtual absence of phosphorylase with the

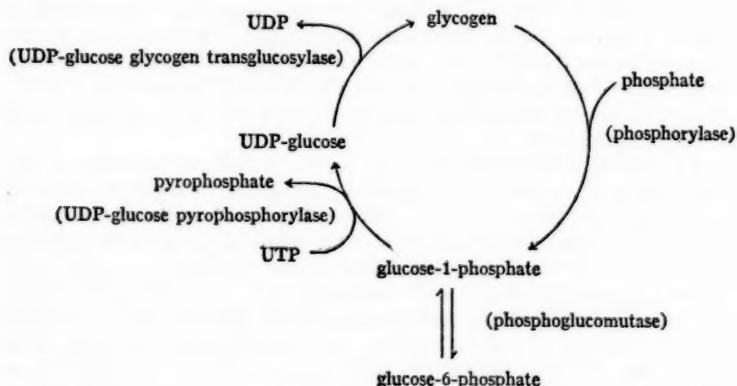


FIG. 1. Glycogen metabolism.

essentially normal content of the other enzymes involved in glycogen metabolism. Both the accompanying glycogen storage and the ability of homogenates to glycolyze hexose phosphates but not glycogen are fully in agreement with the duality of pathways for glycogen synthesis and degradation.

From the above scheme arises an important question of energy expenditure. Glycogen synthesis would be energetically more expensive than formerly assumed, up to a factor of 2, unless pyrophosphate could be effectively recycled. A very logical mechanism would be its utilization for the regeneration of uridine triphosphate out of uridine diphosphate. The importance of recovering the pyrophosphate is still greater in terms of overall glycolysis if the probability of some energy expenditure for the transport across the membrane is taken into account. The net yield of ATP per mole of glucose would drop from 2 to less than 1.

Leloir & Goldemberg (108) have found that the glycogen synthetase of liver homogenates is associated with the glycogen fraction. This fact makes

its purification easy, although the purified enzyme appears to be very unstable. The enzyme is activated by glucose-6-phosphate at physiological concentrations (K_m 6×10^{-4} M). The likelihood of physiological importance as a control mechanism of this activation is obvious. Nothing is known about its mechanism. The effect is not very specific since both glucosamine-6-phosphate and galactose-6-phosphate also activate the enzyme, although with lesser efficiency.

Villar-Palasi & Lerner (109) have observed in insulin-treated muscle a higher glycogen synthetase activity in the absence of added glucose-6-phosphate, although the maximally activated enzyme appears to be the same. They advance the hypothesis of the possible occurrence of two species of synthetase: one active in the absence of added glucose-6-phosphate and the other requiring it for activity. This insulin effect could then be interpreted as an interconversion between the two species. It would be very important in this respect to know something about the nature of the glucose-6-phosphate-activating effect. To emphasize further the need for more study of the factors involved in the actual activity of the enzyme, the recent finding of a considerable activation by sulfate ions, non-additive with that by glucose-6-phosphate [Lerner (110)], is of interest.

Another open problem is the over-all kinetics of the phosphoglucomutase UDP-glucose pyrophosphorylase system. The reported levels of glucose-1-phosphate (111) are fairly low and at the same time relatively high with respect to those of glucose-6-phosphate, if both the equilibrium of the phosphoglucomutase and the direction of its net activity during insulin-activated glycogen formation are taken into account. If the observed concentrations of glucose-1-phosphate had been affected by phosphorolysis during the handling of the tissue and the actual ratio glucose-1- to glucose-6-phosphate during glycogen synthesis were as would be expected, the actual concentrations of glucose-1-phosphate would be so low as to make the affinity of the pyrophosphorylase for it likely to be of great importance.

Galactose-2- 14 C fed to rats raises liver glycogen with much less randomization of its 14 C than occurs with glucose-2- 14 C, an observation that is consistent with the UDP-glucose-mediated pathway for glycogen synthesis [Sin & Wood (112)].

Some advances have also been made on the phosphorylase system. Krebs, Graves & Fischer (113) have observed that phosphorylase-*b* kinase can be obtained from rabbit muscle in a form that is inactive at pH 7.0 or lower. The kinase can be activated by incubation with either Ca^{++} ions or ATP-Mg. The latter activation is enhanced in the presence of added adenosine-3', 5'-phosphoric acid. The authors speculate on the possibility that phosphorylase-*b* kinase could exist in phosphorylated and dephosphorylated forms, active and inactive in a manner analogous to phosphorylases-*a* and -*b*. Muscle phosphorylase phosphatase has been found to act on some phosphopeptides derived from phosphorylase-*a*, but not on other serine-phosphate-containing proteins or peptides (114). The phosphorylase system of

lobster muscle, studied by Cowgill (115, 116), seems to be similar to that in mammals, with the possible exception of a third form of phosphorylase, phosphorylase- ϵ , which seems to require a very high concentration of adenosine monophosphate for activity.

Mansour and co-workers (117) have found a new agent, serotonin, which can increase the formation of cyclic 3,5-adenosine monophosphate and the subsequent phosphorylase activation. Working with *Fasciola hepatica* and *Ascaris lumbricoides*, they have obtained evidence suggesting that serotonin might have a function in invertebrates similar to that of epinephrine in higher animals.

Following the identification of pyridoxal-5'-phosphate as coenzyme of the phosphorylase, Illingworth *et al.* (118) have found that the total phosphorylase activity of the skeletal muscle of rats maintained on a pyridoxine-deficient diet is lower than normal, although both the phosphorylase- α activity and the glycogen content were normal.

Ascites tumors have negligible phosphorylase activities. They have ATP-dependent phosphorylase-activating enzymes but have negligible amounts of dephosphophosphorylase [Nirenberg (119)].

Amylase has been demonstrated to occur in isolated liver cells. It could intervene in the synthesis of glycogen by multiplying the number of molecules that could act as primers [McGeachin & Potter (120)].

Phosphoglucomutase has been further studied by Najjar and co-workers. The phosphoenzyme is activated by preincubation with Mg^{++} ions and imidazole (121). The yeast enzyme is similar in its properties and mechanisms of action to the muscle enzyme (122). An analogous enzyme, a β -glucose-1-phosphate mutase, has been identified in *Neisseria* by Ben-Zvi & Schramm (123).

Emden-Meyerhof glucolytic pathway.—The inhibition of brain hexokinase by glucose-6-phosphate might be decreased by moderate concentrations of inorganic phosphate according to Tiedemann & Born (124). A hexokinase of unusually broad specificity, which seems to phosphorylate galactose in the 6 position almost as fast as glucose, has been reported to occur in *Aspergillus parasiticus* [Davidson 125)]. On the other hand, a typical hexokinase similar to that of yeast has been characterized in *Aspergillus oryzae* [Ruiz-Amil & Sols (126)].

The phosphohexose isomerases have been usually neglected on the assumption that they were in such excess that the isomerization of hexose phosphates could never be a limiting step in glycolysis. Recent findings make it apparent that this neglect is no longer justifiable (see 127). Erythrose-4-phosphate, a normal metabolite, is a very strong competitive inhibitor of phosphoglucose isomerase, which has nearly 100 times greater apparent affinity for it than for its substrates [Grazi *et al.* (128)]. This observation, when related to the fact that the enzyme is competitively inhibited by sorbitol-6-phosphate [Parr (129)] but not by 1,5-sorbitan-6-phosphate [Ferrari *et al.* (130)], leads to the conclusion that the substrates are the open

forms of the hexose-6-phosphates. Obviously then, in this case the real affinity for the substrate is considerably greater than it appears. This helps to explain why, in a careful re-examination of the Michaelis constants, Kahana and co-workers (131) obtained values drastically smaller than those previously reported; their K_m for fructose-6-phosphate was as low as 1×10^{-8} M. On the other hand, since in glycolysis, either from glucose or from glycogen, glucose-6-phosphate is formed in the cyclic pyranose form, the fact that the substrate for the phosphoglucose isomerase is the open form implies the necessity of an intermediate step of ring opening. Whether this reaction can proceed fast enough never to be limiting is open to question. Perhaps there is some mutarotase for glucose-6-phosphate, as formerly found to be the case for glucose [see Bentley & Bhate (132, 133)]. Furthermore, since aldolase must act on the open form of fructose-1,6-diphosphate, the assumption that phosphofructokinase would act on the predominant furanose form of fructose-6-phosphate [Villar-Palasi & Sols (134)] is now open to question. In any case, the over-all kinetics of the various pathways that cross the hexose-6-phosphates stage, as well as the kinetics of the individual enzymes acting on this stage, must be affected by the problem of opening and closing, and the relative proportions at equilibrium of open and closed forms.

Phosphoglucose isomerase has been isolated in apparently pure form from brewers' yeast by Noltmann & Bruns (135). The purified enzyme has a molecular weight of 145,000 and a turnover value of 36,000 moles/mole/min. at 25°. The stereospecificity of the sugar-phosphate isomerase reactions has been investigated by Rose & O'Connell (136).

Drechsler, Boyer & Kowalsky (137) have observed that treatment of muscle aldolase with carboxypeptidase leads to a rapid decrease in activity toward fructose-1,6-diphosphate to about 7 per cent of the original activity accompanying the removal of 3 moles of tyrosine and 2 moles of alanine per aldolase molecule, without apparent change in gross molecular structure. Curiously, the decrease in activity toward fructose-1,6-diphosphate is not accompanied by loss of activity toward fructose-1-phosphate, despite the fact that competition experiments seem to confirm that the same binding site is involved with both substrates. The K_m is somewhat lower for both substrates.

Rieder & Rose (138), in a study of the triosephosphate isomerase reaction in the presence of tritiated water have obtained results that rule out a hydride shift as a mechanism for this isomerization and support the formation of an enolate anion bound to the enzyme.

Glyceraldehyde-3-phosphate dehydrogenase is strongly and specifically inhibited by D-threose-2,4-diphosphate [Racker *et al.* (139); Fluharty & Ballou (140)]. The inhibition is non-competitive with respect to glyceraldehyde-3-phosphate but competitive with the structurally more closely related glycerate-1,3-diphosphate. The apparent K_i is as low as *ca.* 2×10^{-7} M. The characteristics of this inhibition offer important clues to the mechanism

of action of the enzyme. Because of its very high affinity, it might be assumed that, if any threose-diphosphate could be formed intracellularly, it could strongly affect the activity of the dehydrogenase. A plant NADP-linked³ triosephosphate dehydrogenase is also inhibited by threose-diphosphate [Park *et al.* (141)].

Fernández & Grisolia (142) have purified another 2,3-diphosphoglyceric acid-independent phosphoglyceric acid mutase from rice germ. The crystalline phosphoglyceric acid mutases from skeletal muscle and yeast are phosphorylated by incubation with glyceric acid diphosphate. The muscle phosphoenzyme is fairly similar to the phosphoglucomutase of the same source [Pizer (143)].

The heterogeneity of lactic dehydrogenases is proving to be surprisingly wide. In addition to clear-cut differences in substrate specificity and coenzyme requirements, there is increasing evidence of a variety of molecular types that can be resolved by physicochemical techniques. With the occasion of the lactic dehydrogenases, Markert & Møller (144) have proposed the graphic name of "isozymes" for the different molecular types of an enzyme, and Kaplan *et al.* (145) have shown the usefulness of coenzyme analogues for studying the evolution, classification, and differentiation of enzymes. Only passing mention can be made of recent work on lactic dehydrogenases of animal tissues (146 to 148) and microorganisms (149 to 152).

Inhibition by glucose analogues.—Considerable interest is centered in the use of 2-deoxyglucose as metabolic inhibitor *in vivo* [Laszlo *et al.* (153); Brown & Bachrach (154); Bernstein & Black (155)] and as acceptor *in vitro* [van Eys & Warnock (156); Aldridge (157)]. Difficulties in the interpretation of the mechanisms of inhibition have been pointed out by Nirenberg & Hogg (158) and Sols *et al.* (159). Indirect evidence pointing to inhibitory effects beyond the 2-deoxyglucose-6-phosphate stage is strengthened by the observation that 2-deoxygalactose-1-phosphate can strongly inhibit the utilization of galactose by trapping of the uridine coenzyme [Heredia *et al.* (28)].

COMPLEMENTARY AND ALTERNATIVE PATHWAYS

The Embden-Meyerhof pathway is the main route of glucose metabolism in most tissues. Other pathways of glucose metabolism are usually referred to as alternative pathways. In addition, there are metabolic pathways which link other sugars to the main backbone of the Embden-Meyerhof pathway. These can be considered as complementary pathways. Outstanding examples are the pentose phosphate pathway for the oxidation of glucose-6-phosphate and the Leloir pathway for the conversion of galactose to glucose-phosphate.

³ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH₂), for diphosphopyridine nucleotide, reduced form (DPNH); and nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN).

The distinction is not always clear. In fact, a given pathway may work both ways. Thus, the Leloir galactose pathway, when working in reverse for the synthesis of galactose, becomes an alternative pathway of glucose metabolism. For this reason, complementary and alternative pathways are treated in a single section.

Much work has been done and is being done on the ascertainment of the quantitative contribution of different pathways of glucose metabolism in different tissues or organisms and in different conditions. This has referred mostly to the alternative pentose phosphate pathway versus the Embden-Meyerhof pathway. Major emphasis has been given to the results with glucose labelled with ^{14}C in various positions. Recent critical developments indicate that this approach is far from safe. The reviewer refers to the authoritative critical analysis recently published by Katz & Wood (160), which concludes: "It is evident that the calculations of the pathways of glucose metabolism are fraught with many sources of error and such calculations should be interpreted with great caution. Information on the validity of the assumptions used in making the calculations will be necessary in order to make the determinations more reliable."

From the enzymatic point of view, knowledge of the actual working of alternative pathways has been hindered by the fact that much emphasis has been placed on the qualitative and little so far on the quantitative. Frequently the knowledge of what can happen is not accompanied by information on whether it is likely to happen to a major extent. It is natural for the qualitative to be first, but it is unfortunate that there is a tendency to stop at it. The knowledge of the relative amounts and kinetic properties of enzymes of alternative pathways is pathetically low in contrast to the variety that have been shown to occur.

Pyridine nucleotide-dependent reduction and interconversion of sugars.—In addition to the few cases previously known of interconversion between sugars and polyalcohols involving pyridine nucleotides, several new ones have been recently identified. Several general conclusions can be drawn from the accumulated knowledge. The specificity appears to be strict with respect to either aldose or ketose and to either nicotinamide-adenine dinucleotide phosphate (NADP) or nicotinamide-adenine dinucleotide (NAD);³ the aldose reductases usually are NADP-dependent and the ketose reductases, NAD-dependent. Otherwise substrate specificity varies from fairly high, like sorbitol dehydrogenase⁴ (161), mannitol dehydrogenase (163), ribitol dehydrogenase (164), and xylose reductase (165, 166), to such a broad specificity as to cover virtually any aldose and even certain other aldehydes, such as the "aldose reductase" identified by Hers (167) in sheep seminal vesicles and which seems to occur also in rat lens (168). In some cases, two

⁴ Sorbitol dehydrogenase has an apparent affinity for L-erythrulose about 10 times greater than for fructose (162), but the affinity is much smaller if the proportion of open form in fructose solutions is taken into account. The rate is faster with fructose.

coupled reductases can cause a polyalcohol-mediated sugar isomerization aldose \rightleftharpoons ketose (165, 166) or even interconversion D-ketose-L-ketose. From this range of properties it appears that the pyridine nucleotide-dependent sugar reductases can have three different physiological roles. In certain cases, they would be typical complementary pathways of carbohydrate metabolism and, thus, enable the incorporation into a major pathway of an otherwise non-metabolizable sugar (165, 166) or polyalcohol. They could also serve as an additional or alternative hydrogen acceptor (163). Finally, they could mediate an interconversion between reduced NADP and oxidized NAD (165) or vice versa. More than one role can be played by a given system in different conditions. For instance, the well-known sorbitol dehydrogenase of the liver conceivably can either enable the utilization of sorbitol or serve as a hydrogen acceptor in fructolysis, depending upon prevailing conditions. This possibility of alternative or fluctuating roles makes it difficult to obtain an unequivocal interpretation of the physiological meaning of certain cases of accumulation of a sugar or polyalcohol, like the fructose and sorbitol in semen [King & Mann (161)]. The formation of fructose in the seminal vesicles might serve a purpose for the energy metabolism of the spermatozoa or could be a by-product of the metabolism of the seminal vesicles. The sorbitol is more likely to be a by-product of sperm fructolysis.

Fructose.—Although most tissues utilize fructose by the Embden-Meyerhof pathway, there is a well-known instance of an alternative pathway in liver, initially developed by Hers. Lamprecht *et al.* (169) have recently clarified the last steps involved in the metabolism of the glyceraldehyde moiety which results from the splitting of fructose-1-phosphate: oxidation to glycerate and phosphorylation of the latter to glycerate-2-phosphate, thus by-passing most of the enzymes of the Embden-Meyerhof pathway. The same or a similar pathway is likely to occur in intestinal mucosa (57, 58, 59) and in mammary gland [Luick *et al.* (170)]. The utilization of fructose by skeletal muscle is a puzzling question. The fructokinase activity of muscle extracts has been shown to be only a marginal activity of the phosphofructokinase, too small to account for fructose utilization [Villar-Palasi & Sols (134)], and muscle hexokinase is known to have less affinity for fructose than for glucose. Nevertheless, fructose seems to be utilized more efficiently than glucose by muscle in humans (91).

Mannose.—Mannose is the sugar most closely related to glucose. The early assumption that its metabolic utilization was nearly equal to that of glucose has hindered progress in the knowledge of its metabolism. Recent work indicates that this neglect is no longer justifiable. The enzymatic basis of mannose toxicity in the honey bee has been shown to depend on the absence of phosphomannose isomerase, which causes a lack of balance with the hexokinase, which phosphorylates mannose more efficiently than glucose. Competition at the phosphorylation level plus accumulation of mannose-6-phosphate can fully account for the strong toxicity [Sols, Cadenas & Alvarado (127)]. Preliminary observations in the author's laboratory indicate

that mannose can also have some toxicity in mammals, presumably because of phosphomannose isomerase deficiency in certain tissues.

A phosphomannomutase, likely to be involved in the synthesis of mannans, has been identified in yeast by Glaser *et al.* (171). A mannose isomerase seems to occur in *Pseudomonas* (172).

Galactose.—Further information on the Leloir pathway for galactose utilization has been obtained by Kalckar and collaborators in a systematic study of enzyme activities, accumulation of metabolites, and galactose sensitivity in *E. coli* mutants (173, 174, 175). Galactose is inert for the galactokinase-less, bacteriostatic for the galactose-1-phosphate transuridylyase-less, and bacteriolytic for the UDP-galactose 4-epimerase-less mutants.

The substrate specificity of yeast galactokinase has been characterized by Alvarado (176). The K_m for galactose is 3×10^{-4} M. Changes at the level of C2 can be compatible with phosphorylation (2-deoxygalactose, galactosamine, talose), although with considerable decrease in affinity. Changes at C3, C4, and C6 (including L-arabinose and D-fucose) result in compounds inert for the enzyme. Neufeld, Feingold & Hassid (177) have found that in mung bean seedlings there is a galactokinase and an L-arabinose kinase, the respective products being α -galactose-1-phosphate and β -L-arabinose-1-phosphate. For the equilibrium constant of the galactokinase reaction, a value of 25 has been found in the presence of excess Mg^{++} by Atkinson, Johnson & Morton (178). In *Aspergillus parasiticus* galactose is phosphorylated in position 6 by a hexokinase (125).

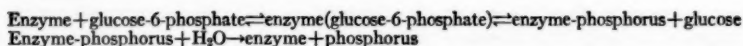
The galactose-1-phosphate uridyl transferase has been studied with a purified preparation from *E. coli* by Kurahashi & Sugimura (179). K_m values for galactose-1-phosphate, glucose-1-phosphate, UDP-glucose, and UDP-galactose were estimated to be 4.1, 2.4, 1.5, and 2.5×10^{-4} M, respectively. In the direction of UDP-galactose formation, the equilibrium constant of the reaction is 1.1. The enzyme requires cysteine for activity.

Yeast UDP-galactose 4-epimerase, which differs from the mammalian enzyme in not being inhibited by nicotinamide-adenine dinucleotide, reduced form ($NADH_2$), has been purified by Maxwell & de Robichon-Szulmajster (180) and was found to have protein-bound NAD. The enzyme-NAD complex can be resolved by treatment with *p*-chloromercuribenzoate. Reactivation of the apoenzyme was obtained with NAD but not with $NADH_2$. Elder *et al.* (181) have obtained evidence that menthol, like progesterone, can increase the rate of oxidation of galactose by liver slices through inhibition of the aldehyde dehydrogenase and the subsequent lowering of the inhibitory $NADH_2$. The mechanism by which menthol stimulates galactose oxidation in galactosemic subjects remains to be explained.

Defective lactose utilization in children can occur without disturbance of galactose metabolism by a lack of β -galactosidase in the intestine; this condition has been described as hereditary alactasia by Holzel, Schwarz & Sutcliffe (72).

Glucose-6-phosphatase.—Hass & Byrne (182) have found that the hepatic

glucose-6-phosphatase can catalyze the exchange of ^{14}C -glucose with the glucose moiety of glucose-6-phosphate. The results are consistent with the mechanism:



K_m and K_i values for glucose of 6×10^{-3} and 9×10^{-3} M, respectively, were obtained. Phosphate is a competitive inhibitor with a K_i of 2×10^{-2} M. No difference was found between the kinetic parameters of the enzyme prepared from normal and diabetic muscles.

Little or no enzyme occurs in foetal liver in mammals. In the developing chicken embryo, hepatic glucose-6-phosphatase activity appears almost simultaneously with the appearance of the first hepatic cells [Kilsheimer *et al.* (183)].

Pentose phosphate pathway.—Stern & Vennesland (184) have shown, using deuterium as a tracer, that the glucose-6-phosphate and 6-phosphogluconic dehydrogenases cause direct hydrogen transfer from the substrate to the β -para position of the nicotinamide ring of NADP.

There is now considerable interest in the pathological aspects of glucose-6-phosphate dehydrogenase deficiencies, particularly in erythrocytes. It is of particular interest that, while the widely distributed primaquine-sensitive hemolytic anemia seems to be related to a marked deficiency in enzyme content without noticeable qualitative changes, Kirkman *et al.* (185) have found in a case of congenital non-spherocytic hemolytic anemia that the glucose-6-phosphate dehydrogenase differs qualitatively in a smaller affinity for glucose-6-phosphate (K_m about five times greater) and apparently in a considerably greater lability. Moreover, Rimon *et al.* (186) have obtained an indication of the existence of an activating factor, probably of an enzymatic nature, in the stroma of normal erythrocytes, which is absent in the primaquine-sensitive subjects.

Bonsignore and collaborators (187) have shown that the non-oxidative pathway of pentose phosphate synthesis from hexose monophosphate does not initially require triose phosphate. There is a coupled attack of transketolase and transaldolase upon two moles of fructose-6-phosphate, the former producing erythrose-4-phosphate and the latter glyceraldehyde-3-phosphate, each of these cleavage products serving as acceptor for the other enzyme. Orthophosphate inhibits both enzymes. The inhibition of transaldolase is competitive with fructose-6-phosphate and non-competitive with sedoheptulose-7-phosphate or glyceraldehyde-3-phosphate. The K_i in either case is *ca.* 5×10^{-2} M (188). Venkataraman, Datta & Racker (189) have reported evidence suggesting the formation of a dissociable dihydroxyacetone enzyme in the transaldolase-catalyzed reaction.

Pricer & Horecker (190) have studied the deoxyribose aldolase from *Lactobacillus plantarum*; it catalyzes a reversible cleavage of 2-deoxyribose-5-phosphate to yield acetaldehyde and D-glyceraldehyde-3-phosphate. The affinity of the enzyme for all three substrates is similar, the K_m being *ca.* 10^{-3} M.

The relative contributions to pentose biosynthesis of the oxidative and the non-oxidative reactions of the pentose phosphate pathway in different tissues of mammals have been investigated by Hiatt & Lareau (191). In most tissues, but not in muscle, the non-oxidative reactions seem to predominate.

Levin & Racker (192) have purified the 2-keto-3-deoxy-8-phosphooctonic acid synthetase from *Pseudomonas aeruginosa* and found that it condenses phosphoenol pyruvate with arabinose-5-phosphate rather than with ribose-5-phosphate as it formerly appeared to do.

Glycerophosphate pathway.—Recent work is making it apparent that in many tissues the NAD-linked α -glycero-phosphate dehydrogenase is an important complementary or alternative pathway for the classical NAD-regenerating role of lactic acid dehydrogenase. Outstanding is the case of the muscle of flying insects, in which the studies of Sacktor and of Bücher and co-workers have clearly established that not only is the dihydroxyacetone-phosphate reduction the major NADH_2 acceptor in glycolysis but also that, by a coupling with a mitochondrial glycero-phosphate oxidase, the α -glycero-phosphate acts in a cyclic mechanism which mediates the transfer of hydrogen across the barrier between the cytoplasm and the mitochondria (193 to 196). An outstanding exception appears to be the case of most tumors, in which the α -glycero-phosphate dehydrogenase is very small, both absolutely and with respect to the lactic acid dehydrogenase [Delbrück *et al.* (197); Boxer & Shonk (198)]. Accordingly, it is in tumors, but not in many other animal tissues, that lactic acid really accounts for most of the anaerobic glycolysis [Ciaccio *et al.* (199)].

Pyruvate to phosphopyruvate.—Recent work on the reversal of the pyruvate kinase reaction in liver quantitatively supports the occurrence of CO_2 fixation [Landau *et al.* (200)]. Nevertheless, Hoberman & D'Adamo have obtained evidence that several pathways are involved, with and without malate and fumarate as intermediates (201). A direct formation of oxaloacetate from pyruvate and CO_2 has been identified by Utter & Keech (202). Pyruvate kinase itself can catalyze the enolization of pyruvate; the spontaneous rate is too slow to account for the formation of phosphoenolpyruvate [Rose (203)].

MISCELLANEOUS

The study of control mechanisms in the field of carbohydrate metabolism is now of considerable interest. Lack of space prevents detailed treatment here. The intrinsic mechanisms of cellular enzymatic regulation have been thoroughly reviewed by Holzer (204). Of the recent literature, two major approaches are represented by the study of enzymatic activities as possible pacemakers in Racker's laboratory [see Wu (205)] and the spectrophotometric study of intracellular glycolysis in Chance's laboratory [see Chance *et al.* (206)] and by Lonberg-Holm (207). The hormonal regulations have been extensively reviewed by Beloff-Chain & Pocchiari (208). In particular, the mechanism of action of insulin is a subject of constant speculation; it has been reviewed by Fisher (84, 209) and Randle & Young (85).

Another type of regulatory mechanism depends on the control of enzyme synthesis. The amount of several glycolytic enzymes in liver can increase by adaptation in response to dietary changes [Fitch & Chaikoff (210); Petrášek (211)]. That tissues of mammals are capable of adaptation to certain uncommon carbohydrates is evidenced by results with tissue cultures [Bradley & Syverton (212); Morgan & Morton (213)]. A repressor theory on the genetic control of inducibility in microorganisms has been developed by Pardee, Jacob & Monod (214) [see also McQuillan *et al.* (215)].

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METABOLISM OF AMINO ACIDS^{1,2}

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NITROGEN METABOLISM

Usually only the grossest aspects of a metabolic process can be understood from studies with the intact organism, whereas a detailed knowledge of the mechanism often quickly follows when the process can be examined in a soluble system. Therefore it is of particular significance that it has finally been possible to obtain cell-free extracts which will fix atmospheric nitrogen. Carnahan and his associates have prepared such extracts from *Clostridium pasteurianum* by using either a Hughes press or anaerobic autolysis at 30° (1 to 4). The enzymes responsible for the fixation are not sedimented at 144,000 x g for 2 hr. Fixation of up to 12 µg. N/mg. protein was secured, almost entirely in the form of ammonia, and an absolute dependence on pyruvate was noted. Pyruvate presumably functions as a metabolizable substrate that yields reducing power. A nitrogen-dependent change in absorption of the extract at 300 to 365 mµ was also reported. The cell-free nitrogen fixation in *C. pasteurianum* has been confirmed and the observations extended to other species, including several blue-green algae and *Rhodospirillum rubrum* (5, 6). It should now be possible to supply answers to a long list of questions that have accumulated with respect to nitrogen fixation in previous years. Some of the amplifications and extensions to that list include the demonstration of a cobalt requirement in symbiotic N₂ fixation in the Lucerne legume (7) as had already been found in the blue-green algae. Further, the inhibition of nitrogen fixation by oxygen, first reported by Meyerhof & Burk, was shown to be competitive by Parker & Scult (8). In an examination of possible candidates for intermediates in nitrogen fixation, it was shown that soybean nodules will reduce N₂O to N₂ (9) and that hydrazine will be taken up by heavy cell suspensions of *Asotobacter vinelandii* (10). In both instances, however, the authors rejected these compounds as possible intermediaries. Chloramphenicol in concentrations that completely inhibit growth reduces nitrogen fixation by only 20 per cent (11).

The investigations concerned with unravelling the mechanism of nitrate assimilation reactions represent the other side of the coin in that these reactions, though demonstrable in soluble systems, are beset with difficulties in interpretation of the physiological electron acceptor or donor, or even of the

¹ The literature survey pertaining to this review was concluded October, 1960.

² The following abbreviations are used: ADP for adenosine diphosphate; ATP for adenosine triphosphate; NADPH₂ for nicotinamide-adenine dinucleotide phosphate, reduced form.

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in vivo substrate. This is strikingly demonstrated by some recent findings by Mager & Weiss-Zehavi (12, 13). During an investigation of what was presumed to be a hydroxylamine reductase in *Escherichia coli*, it was found that the enzyme was capable of being repressed by cysteine or methionine. This led to the discovery that sulfite could also be reduced by the enzyme preparations. No change in the ratio of sulfite to hydroxylamine reductase activity was observed during a 150-fold purification. Both activities were specific for the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH_2),⁴ and a requirement for flavin-adenine dinucleotide could be demonstrated in each case. This evidence that one enzyme was capable of acting on these two markedly different substrates raised the question as to which one was the "true" substrate. The repressibility by the sulfur amino acids and a 100-fold smaller K_m for sulfite militates against hydroxylamine.

Roussos & Nason (14) have described a reduced pyridine nucleotide-hydroxylamine enzyme obtained from soybean leaves. Though purified 200-fold, there was an associated ability to utilize nitrite during the later stages of the purification. The product of both reactions has not been identified, but a requirement for an unknown organic cofactor for both reactions has been established. The *Neurospora crassa* nitrite reductase was purified fiftyfold by Nicholas *et al.* (15) and was shown to be an iron- and copper-containing flavoprotein. There was no stimulation of the purified enzyme by pyridoxal phosphate, nor was there a concentration of pyridoxal phosphate in the purified enzyme preparations, though they were able to confirm the activation, *in vitro*, of the enzyme in a pyridoxine-requiring mutant, as first reported by Silver & McElroy (16). The *Pseudomonas aeruginosa* nitrite reductase, which has been purified more extensively (600-fold), will not function with reduced pyridine nucleotide, but only dyes and flavin can act as electron donors (17). A nitrate reductase has been purified 115-fold from the same organism (18). In this instance, nicotinamide-adenine dinucleotide, reduced form,⁴ is a specific electron donor with a K_m of 4.2×10^{-4} . In this organism, the enzyme is largely in the non-sedimentable fraction, in contrast to the nitrate reductase in *E. coli*, which Itagaki & Taniguchi report as being largely (60 to 70 per cent) particulate (19). The particulate enzyme has been investigated by Heredia & Medina (20), who found stimulation by vitamin K₃ in such preparations.

Nicholas & Jones (21, 22) have partially purified the enzyme that catalyzes an oxidation of hydroxylamine to nitrite in cell-free extracts of

⁴ Editorial note: In accordance with the newly recommended usage [see *Nature* 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH_2), for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH_2), for triphosphopyridine nucleotide, reduced form (TPNH).

Nitrosomonas europaea. This represents the first report of a cell-free system capable of carrying out the reaction. Polyanions such as tungstate, selenate, pyrophosphate and citrate were very inhibitory. Phenazine methosulphate or cytochrome-*c* was used as the electron acceptor.

AMINO ACID PENETRATION

It would appear to be a good rule that whenever cells are dependent on the environment for their supply of an amino acid, the possibility should be assessed as to whether the rate of entrance of the amino acid into the cell is limiting, directly or indirectly, to the process under consideration. Kihara & Snell have proceeded on the assumption that the stimulatory effect of tryptic casein digests on the early growth of *Lactobacillus casei* is attributable to amino acid limitation and that streptogenin plays no special role in the metabolism, other than as a readily available source of limiting amino acids. They have validated this view in two ways. It has been possible to devise a supplement of simple compounds superior to streptogenin (23) for the growth of *L. casei*, and they have set up a model system in which *Leuconostoc mesenteroides* was grown in the presence of two amino acid antagonists. Maximal growth rates could be achieved either with large amounts of both of the two antagonized amino acids or by small amounts of a dipeptide containing both amino acids (24). There have been other recent examples of a peptide having nutritional properties different from the component amino acids (25 to 27). The reversal of penicillamine inhibition of *E. coli* by the branched-chain amino acids (28) and mercaptosuccinic and homoserine reversal of norleucine inhibition of *Vibrio comma* (29) may represent a different facet in which entrance of an inhibitor can be limited.

Jacobs *et al.* (30) have observed a reduction in methionine absorption by the perfused upper small intestine by either deoxypyridoxine or 2,4-dinitrophenol. The 2,4-dinitrophenol inhibition could be overcome by pyridoxal phosphate. Intestinal absorption of amino acids has also been studied by Nathans *et al.* (31) who found a stimulation by glucose.

The urinary excretion of taurine was enhanced upon intraperitoneal administration of β -amino acids (32). This was thought to be attributable to an effect on the tubular absorption of taurine, and represents the first evidence for a taurine- β -amino acid competition. However, it does not appear possible to generalize from one system to another since this competition could not be found in the Ehrlich carcinoma cell (33).

Evidence for amino acid penetration into brain, other than by simple diffusion, is that the L form of tyrosine equilibrates between plasma and cerebrospinal fluid faster than the D form (34) and that the initial uptake of 5-hydroxytryptophan is faster than diffusion (35).

The influence of hormones on amino acid uptake has attracted attention since hormones represent a rather direct way of influencing metabolism. There is an enhanced incorporation of α -amino isobutyric acid by rabbit uterus from an ovariectomized animal if the animal has received estradiol

even 30 min. beforehand. *In vitro* there is no effect, even though the estradiol is taken up by the tissue (36). The efficacy for *in vitro* uptake of anabolic steroids, such as testosterone, administered *in vivo* has also been shown for a levator ani muscle preparation (37). In the case of growth hormone, *in vitro* stimulation of α -amino isobutyric acid penetration by the intact diaphragm preparation was demonstrated by Kostyo *et al.* (38). The metabolic inertness of α -amino isobutyric acid has recommended it for studies of amino acid transport since its entrance into the cell is not complicated by an associated metabolism to other forms. However, it is not always possible to extrapolate from the behavior of this compound to all other amino acids. Manchester & Young could confirm the acceleration, by insulin, of α -amino isobutyric acid accumulation by diaphragm (39), but, of all the other amino acids tried, only glycine was affected in the same manner (40). Hence, these authors do not regard the enhanced incorporation of amino acid by insulin-stimulated diaphragm as being attributable to an increased amino acid transport.

PYRIDOXAL PHOSPHATE, DECARBOXYLATION, AND TRANSAMINATION

The pioneering studies of Metzler & Snell on the catalytic potentialities of pyridoxal phosphate continue to receive verification and amplification. Moreover, with the increasing availability, in highly purified form, of a number of pyridoxal phosphate-dependent enzymes, the properties of enzyme-bound pyridoxal phosphate are now being examined and contrasted with the free compound. Nakamoto & Martell (41, 42) have continued their extensive studies of the ultraviolet absorption of various hydroxypyridine aldehyde derivatives as a function of pH (41, 42). They have summarized the ionic equilibria involved and assigned the absorption bands. These studies should provide considerable help in elucidating the absorption spectra of enzyme-bound pyridoxal phosphate. In the case of the cupric chelates of pyridoxaline and pyridoxylidene valine, Christensen (43) has concluded that extreme lowering (3 pH units) of the pK of the pyridinium nitrogen does not depend exclusively on whether or not a bond is formed between the metal and the phenolate ion but is also a function of the extent of electron displacement occasioned by the bond. A finding of considerable potential utility as an analytical tool is the observation that the carbonyl group of pyridoxal phosphate will react with cyanide to produce a fluorescent compound (44). One of the more complex model systems is the interaction of cystine with pyridoxal phosphate. Pyruvate, ammonia, CO₂, free sulfur, thiosulfate, cysteine sulfinic acid, and alanine thiosulfonate are among the products identified. The postulate that thiocysteine is an intermediate provides a rationalization for some of the products observed (45).

Shukuya & Schwert (46) have prepared the glutamic acid decarboxylase of *E. coli* in a state of 90 per cent purity. The molecular weight is estimated to be 300,000. There are two pyridoxal phosphate residues per mole of apoenzyme. The addition of glutamate to the enzyme causes changes in the absorption spectrum that may be followed conventionally or fluorometrically

(47). The enzyme is more stable when stored at 25° than 0° and, hence, may be added to the list of cold-labile entities (48).

In a preliminary note, King & Brown (49) have found a correspondence between the Michaelis constant for the leucine decarboxylase of *Proteus* and the stability constant for the Schiff base of leucine and pyridoxal phosphate as a function of pH. An improved analytical method for measuring decarboxylases depends on the separation of the amine and the amino acid (50).

In their continuing study of the highly purified glutamic aspartic transaminase, Jenkins &Sizer (51) have recorded the spectra of the pyridoxamine and pyridoxal forms of the enzyme. The interconversion of the two forms upon reaction with substrate was also characterized. Patwardhan (52) was able to resolve partially the glutamic-aspartic transaminase of the bean *Dolichos lablab* and show a dependence on iron.

The isotope-exchange reactions catalyzed by the γ -aminobutyric-glutamic transaminase are of the same nature as have been found for the exclusively α -amino acid transaminases (53). The non-enzymatic exchange transamination between glycine and glyoxalate proceeds so rapidly in the presence of Cu^{++} that it is actually inhibited by pyridoxal phosphate (54).

Kun *et al.* (55) have investigated the action of β -fluoro-oxalacetate on the glutamate-aspartate transaminase of heart mitochondria. It is a competitive inhibitor with a low turnover number. Upon conversion to the presumed β -fluoroaspartic acid, this compound breaks down to oxalacetic acid, hydrofluoric acid, and ammonia.

DEHYDROGENASES

Glutamic dehydrogenase continues to be subjected to intense scrutiny, and well it might since in the absence of other enzymes with sufficient turnover numbers it is forced to play an enigmatic dual role—the primary means of assimilating NH_3 to amino acid nitrogen and vice versa.

Direct evidence has been obtained for the suggestion (56) that glutamic dehydrogenase is a tetramer. In an examination of the crystalline enzyme by electron microscopy (57), spheres with diameters of 125, 90, and 75 Å were revealed; the cubes of these numbers are almost exactly in the ratio 4:2:1. In a thorough study of the kinetics of the beef enzyme, Frieden (58) concluded that there is an obligate sequence of addition of substrate to the enzyme in the order NADPH_2 , NH_4^+ , and α -ketoglutarate. The lack of binding of α -ketoglutarate by the enzyme in the absence of reduced pyridine nucleotide has also been demonstrated by Fisher (59). These findings have wide-ranging implications. The mechanisms of many complex enzymatic reactions are being investigated by looking for partial exchange reactions in the presence of fewer components than are necessary for the over-all reaction. Failure to detect such an exchange may be attributable to an inability to take up one of the essential components because a component, not necessary for the partial reaction but essential to the binding, may be absent.

Nakamoto & Vennesland (60) have demonstrated that when nicotin-

amide-adenine dinucleotide phosphate⁴ is used, the same beta-specificity in the hydrogen transfer is maintained as they had previously found with nicotinamide-adenine dinucleotide. However, the specificity with respect to substrate is not so absolute as had been previously thought (61). Under appropriate conditions, a variety of amino acids can be dehydrogenated. The pH optimum is 9.8 for leucine, as compared to 8.4 for glutamic acid, and L-norvaline is 17 per cent as active at pH 9 as glutamic acid is at pH 8. Since the pH can be so critical, it is of some significance that the pH optimum of the particulate glutamic dehydrogenase of *Lupinus albus* did not change after solubilization, although the *L. albus* aconitase did so (62). Fincham & Bond (63) report the characterization of an altered glutamic dehydrogenase obtained from a *Neurospora crassa* mutant. In addition to being heat-activatable, though more thermolabile than wild type, the Michaelis constants for some of the substrates are as much as thirtyfold different from the enzyme of the parental strain.

The central role of glutamic dehydrogenase in metabolism has prompted an examination of the dehydrogenase from the point of view of hormonal regulation. In a preliminary communication, Yielding and co-workers (64) report that at a concentration of $5 \times 10^{-5} M$ the rat liver enzyme was inhibited 94, 95, and 97 per cent by diethylstilbestrol, estradiol, and progesterone, respectively. ADP could reverse the sterol inhibition. They could not confirm the reported stimulation of the enzyme by corticosterone and cortisol (65). The enzyme from human placenta has been partially purified (66) and appears to have properties similar to those of the beef enzyme.

Pierard & Wiame (67) have carried out a detailed study of the alanine dehydrogenase of *Bacillus subtilis*. Except for substrate specificity, it is remarkably similar to the glutamic dehydrogenase of beef liver—even to the enigma of its function. The observation that a mutant strain of *B. subtilis* which was unable to utilize NH_4^+ for growth lacked glutamic dehydrogenase but not alanine dehydrogenase seemed to confirm the key role of the glutamic enzyme in NH_4 assimilation. However, Hung, Shen & Braunstein (68, 69) have now presented evidence that in the genus *Bacillus* glutamic dehydrogenase is usually absent; of five species examined only *Bacillus anthracoides* contained both enzymes. Furthermore, the inability of strains of *Bacillus subtilis*, which possess only alanine dehydrogenase, to grow on NH_4^+ as nitrogen source disappears if an appropriate carbon source is chosen (70, 71). The block in the NH_4^+ negative mutant of Wiame & Pierard is attributed by Braunstein, not to the lack of glutamic dehydrogenase, but to some other as yet unexplained facet of ammonia assimilation. Goldman (72), who had previously reported an inhibition of the alanine dehydrogenase of *Mycobacterium tuberculosis* by tetracycline, could not confirm this with a purified preparation of the enzyme. He has shown this is attributable to a requirement for Mg^{++} (present only in the cruder preparations), since it is actually the Mg^{++} chelate complex with terramycin that is inhibitory.

OXIDASES

Wellner & Meister (73) have obtained a crystalline L-amino acid oxidase from the venom of *Crotalus adamanteus* after a twentyfold purification. The enzyme has a molecular weight of 128,000, and there are two moles of flavine-adenine dinucleotide per mole of enzyme. These investigators have made the interesting observation that, by bleaching the enzyme with L-leucine anaerobically and then admitting only minute amounts of oxygen, bands at 375, 385 and 475 μ appear. Further addition of oxygen restores the original spectrum. This is interpreted as indicating that there are two steps in the re-oxidation of the reduced enzyme (74). An L-amino acid oxidase has also been partially purified from chicken liver. The enzyme was tightly bound to the microsomes. It could attack basic amino acids but not L- α -hydroxy acids (75).

Through iron has been found in association with the D-amino acid oxidase, removal leaves the activity unimpaired (76). A kinetic approach to the nature of the active site of D-amino acid oxidase suggests that an SH group is involved in binding the adenylic moiety of flavin-adenine dinucleotide to the enzyme (77), and the isoalloxazine ring interacts with the protein at two sites (78, 79). Observations on inhibition of the enzyme by benzoate (80) and chlorpromazine (81) and on various antibiotics as competitors of the dinucleotide (82) have also been made.

Falcone *et al.* (83) have reported the induction of spore germination in *Bacillus subtilis* by H_2O_2 as well as L-alanine. The triggering action of L-alanine is well-known, and the conversion of the L-alanine to pyruvate and ammonia is established (84). If it could be shown that this metabolism proceeds through an L-amino acid oxidase with concomitant H_2O_2 production, these findings would interlock nicely.

The diamine oxidase of pea seedlings has been further purified. The copper content increases with purification and reaches 0.08 per cent in the best preparations (85). Zeller *et al.* (86, 87) have concluded that the active center of monoamine oxidase contains an acceptor site for the alpha-carbon and a site with an unshared pair of electrons for the alpha-hydrogen. The inhibition of this active center is remarkably dependent in a periodic manner on the chain length of aliphatic primary alcohols (88). Green & Haughton (89) have developed a new assay for monoamine oxidase; it consists of trapping the aldehyde as a semicarbazone and then converting this to the 2,4-dinitrophenylhydrazone for estimation. Weissbach *et al.* (90) have devised a spectrophotometric assay which uses kynuramine as substrate.

REGULATION

The phenomena of "negative feedback" inhibition of enzyme activity and repression of enzyme synthesis are well-documented control mechanisms in microorganisms. Evidence continues to accumulate that these mechanisms are also manifested in the higher animals. Walker (91) has demonstrated that

a diet containing 5 per cent creatine will reduce in rats the level of the first enzyme in creatine biosynthesis (arginine-glycine transaminidase) to one-fifth that of control animals. Removal of the creatine from the diet results in a restoration of the activity to normal values. *In vitro*, the enzyme is not inhibited by creatine. *In vivo*, creatinine does not substitute for creatine as a repressor.

Results of the same general nature have been obtained with the chick, though here the repression is even more dramatic (92). Fitch *et al.* (93) have confirmed the findings for the rat. These new observations are of the level of demonstrating changes in enzyme activity. As yet, no experiments have confirmed the enzyme activity is here equivalent to enzyme concentration. Two sour notes have been struck on this point in other systems: Kenney (94) observed that the adrenocorticoid-induced increase in tyrosine transaminase activity in rat liver is not accompanied by an increased amino acid incorporation into protein precipitable by antisera prepared with highly purified enzyme. In the tryptophan pyrrolase system it has been found that the enzyme activity in uninduced preparations can be increased several-fold by the addition of a microsomal heat-stable factor (95), and the suggestion is made by Greengard & Feigelson that substrate induction, here, may be in part intracellular translocation of the activator rather than *de novo* synthesis. If this turns out to be the entire explanation, it will be difficult to understand, among other things, how 8-azaguanine could inhibit tryptophan pyrrolase induction (96).

Civen & Knox (97) have used the adrenalectomized rat to study the action of tryptophan analogues on pyrrolase induction independent of the effects on adrenal corticoid levels. However, corticoids are not always factors to be considered, since in the case of the rise in renal glutaminase induced by dietary ammonium chloride they had no effect (98). Glutamotransferase increases dramatically during fetal development (99), whereas for 5-hydroxy-tryptophan decarboxylase (100) and tryptophan pyrrolase (101) a sudden rapid increase sets in on parturition. Nemeth has made an interesting point concerning enzymes that appear after parturition. Since they seem to play no role in fetal development, they are prime sites for "inborn errors of metabolism" for their loss can be of no consequence until after birth.

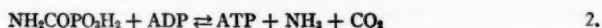
In the bacterial systems, control mechanisms of increasing complexity and their subtle concomitants are being discovered and encountered. Halpern & Umbarger (102) have provided evidence for two acetolactate-synthesizing systems in *Aerobacter aerogenes*. One appears in response to growth under acid conditions, is unaffected by valine, and is regarded as functioning primarily to produce the acetoin that is characteristic of growth of this organism under acid conditions. The other system, which predominates under neutral growth conditions, assures a supply of acetolactate for valine biosynthesis but, since its activity can be inhibited by valine, will not wastefully provide that supply in the presence of exogenous valine. These two means of producing acetolactate provide for increased versatility in responding efficiently to the environment.

Moyed (103) has described an example of false feedback inhibition. 5-Methyltryptophan is shown to cause a feedback inhibition of tryptophan biosynthesis before anthranilic acid, and, since 5-methyltryptophan cannot substitute for tryptophan, growth ceases. The false feedback principle is also invoked to explain the transitory bacteriostatic action of 2-thiazole alanine (104). The analogue so reduces histidine synthesis that the system becomes unrepressed, and the increased enzyme synthesis then more than makes up for the reduction in activity.

Doy & Pittard (105) have demonstrated that tryptophan can inhibit at a step before anthranilic acid and therefore regard Moyed & Friedman (106) as incorrect in assuming that inhibition occurs after anthranilic acid. In principle, there is no reason why the terminal product may not inhibit at several points in a long, highly branched pathway, and both authors may be correct. Control mechanisms for methionine and cysteine synthesis have been described (107), encountered in actinomycin synthesis (108), and sought for in *Micrococcus glutamicus* (109).

UREA FORMATION

There seems little doubt that, though the mechanism, stoichiometry of formation, and cofactor requirements are different for carbamyl activation in bacteria and ureotelic animals, carbamyl phosphate serves as the intermediary in both types of organisms. Caravaca & Grisolia (110) have offered further evidence to support these points, including a very neat experiment showing that a combination of carbamyl phosphate synthetase and carbamyl kinase acted as an ATPase, thus proving the non-identity of the enzymatic reactions but the identity of the intermediate.



However, the role of N-acetyl glutamate in Reaction 1 is still not understood. It might be structurally necessary for an active enzyme but might play no chemical role in the reaction, which is indeed the case for most, and often all, of the amino acids of an enzyme. On the other hand, it might be a primitive vitamin covalently participating in the over-all reaction, possibly only after conversion to the correct coenzyme form. Certainly, the finding that two moles of ATP and a cofactor are involved suggests an intermediate before carbamyl phosphate. Jones & Spector (111) have provided evidence that this intermediate might be an activated form of CO_2 , since ^{18}O -labelled CO_2 can contribute to the inorganic phosphate formed at pH 8.9. This observation had been missed by Reichard (112) and Metzenberg *et al.* (113), since their experiments had been done at pH 8.0 where the rapid exchange of CO_2 with water obscured the results. A series of glutamic acid derivatives were tried as replacements for N-acetyl glutamic by Grassl & Bach (114). Among the unsuccessful candidates were N-acetyl-L-glutamine and N-acetyl-L-isoglutamine. Advantage has been taken of the ease of converting inorganic

phosphate to carbamyl phosphate chemically as a means of preparing terminally labelled ATP through reversal of carbamyl kinase (115, 116).

Since carbamyl phosphate also functions in pyrimidine biosynthesis, carbamyl phosphate synthetase should be present in tissues that can synthesize pyrimidines even if they do not form citrulline. This has been shown for rabbit intestinal mucosa (117). The possibility of endogenous bacteria being responsible for the carbamyl phosphate synthesis was ruled out since there was an N-acetyl-glutamate requirement.

Mistry & Grillo (118) found no difference in the level of the ornithine or aspartyl transcarbamylase in biotin-deficient and normal rats. Moreover, these enzymes were not inhibited by avidin. This lack of a biotin effect in the rat differs from the positive finding in bacteria, although the latter is now regarded as an indirect effect since a mixture of peptides will replace the biotin requirement for maximal transcarbamylase activity in *Streptococcus lactis* 8039 (119).

A detailed paper has now appeared on argininosuccinic aciduria (120). Excretions of up to 7 gm. of argininosuccinic acid per liter of urine were obtained. A puzzling observation was the finding of a threefold higher concentration of the argininosuccinate in the cerebrospinal fluid as compared to serum. This implies that extrahepatic tissues may possess all or parts of the urea cycle. Evidence in support of urea synthesis from arginine in brain has appeared (121). The claim has also been made that isolated purified rat hearts will form urea from ammonia (122).

Bach & Killip (123) have prepared crystalline arginase from sheep and horse liver by a procedure similar to that successfully used with ox liver. The three enzymes have a pH optimum of 9.2 and a K_m of 7×10^{-4} . Brown & Cohen (124) have assayed the livers of 23 representative vertebrate species for their content of urea cycle enzymes. They have incorporated their findings into a scheme of vertebrate evolution. The early piscine ancestors of man are regarded as ureotelic, and the partial or complete loss of the enzymes associated with urea formation accompanied the appearance of evolutionary branches that became uricotelic (birds, lizards) or ammonotelic (teleost fishes).

ALANINE, ASPARTIC ACID, AND GLUTAMIC ACID

Hird & Morton (125) have investigated the oxidation of L-alanine by rat liver mitochondria. α -Ketoglutarate was able to effect an eighteenfold stimulation of the conversion of isotopically labelled L-alanine to CO_2 . Low concentrations of pyruvate were also stimulatory. These observations have been interpreted to favor transamination rather than oxidation via an L-amino acid oxidase as the first step in L-alanine utilization.

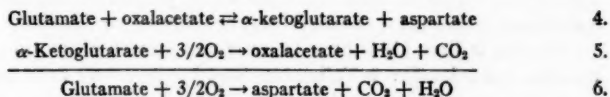
Transamination with pyruvate as acceptor has been studied in different animal species. In general, these reactions are high in small mammals, and the specificity with respect to amino donor is repeated in the different species (126). *Clostridium propionicum* also utilizes this type of transaminase since the amino group of β -alanine is transferred to pyruvate in the course of its

metabolism (127). The enzyme responsible for β -alanine formation from pyrimidines in *Clostridium uracilicum* has been purified by Campbell (128). In contrast to the less specific mammalian enzyme, it will attack N-carbamyl β -alanine but is inactive with N-carbamyl β -amino isobutyric acid.

Aspartic acid.—The ease with which the amino acids related to glycolysis and the tricarboxylic acid can be synthesized from carboxylic acids can be seen in experiments with isolated rat diaphragm (129). In these experiments, of all the protein amino acids, only alanine, aspartate, and glutamate incorporated isotope when it was administered as citrate, succinate, isobutyrate, propionate or acetate. The author postulates a reversal of the α -ketoglutarate dehydrogenase reaction in order to explain the appearance of label in glutamic acid from succinic acid. This is hardly necessary since succinate-2- ^{14}C was employed and the tricarboxylic acid cycle was functioning. In *Staphylococcus aureus* (Duncan), although uniformly labelled glucose leads to radioactive aspartic acid, the glutamic acid is unlabelled. In this instance the tricarboxylic acid cycle is incomplete (130). The growth of this organism can be stopped by rapid passage of CO_2 -free air through the medium, and this can be reversed by the addition of aspartic acid.

De Groot & Lichtenstein have studied the action of *Pseudomonas fluorescens* and rat liver extracts on α -N-alkyl derivatives of asparagine. The bacterial extracts attack these derivatives (131), whereas they inhibit the asparaginase and β -aspartyl transferase activities of rat liver (132). The similarity in the pattern of inhibition of both these activities is offered as evidence that they are to be attributed to a single enzyme.

Glutamic acid.—Krebs & Bellamy (133) have studied the oxidation of glutamic acid in homogenates of rat and pigeon liver, pigeon breast muscle, and sheep kidney cortex. Under appropriate conditions there is an accumulation of aspartic acid almost equal to the disappearance of glutamate. This is offered in support of the following scheme:



Borst & Slater (134) have made equivalent observations with rat heart sarcosomes. These findings militate against the notion that glutamic dehydrogenase plays an important role in glutamic acid oxidation.

When highly purified preparations of glutamine synthetase from sheep brain are heated in the presence of glutamate, ATP, and Mg^{++} , small amounts of pyrrolidone carboxylate are obtained (135). In view of the greater tendency of γ -glutamyl derivatives to cyclize, this can be interpreted in terms of such an intermediate bound to the enzyme. The optical specificity of glutamine synthetase has been investigated in great detail by Varner (136). While conditions can be found in which the D-isomer is used very effectively, the greatest specificity would be exerted under *in vivo* conditions. While many cell lines require glutamine in tissue culture, this is not true of chick heart.

The glutamine synthetase of hen heart has been partially purified and studied by Gothoskar *et al.* (137).

VALINE, ISOLEUCINE, AND LEUCINE

Cell-free systems from yeast, *E. coli*, and *N. crassa* are being used to validate the role of various intermediates in the biosynthesis of valine and isoleucine. The yeast system has been divided into two fractions, one of which will convert α -acetolactate to α,β -dihydroxyvaleric acid (138) and the second of which will convert the dihydroxy acid to α -ketoisovaleric acid (139). Umbarger *et al.* (140) have shown the same conversions in *E. coli* and have also examined the analogous reactions in the isoleucine pathway starting from α -aceto- α -hydroxybutyrate. *E. coli* contains two enzymes which will form α -acetolactate and α -aceto- α -hydroxybutyrate with pH optima at 6.0 and 8.0, respectively (141). These enzymes utilize pyruvate to form an active acetaldehyde group on thiamine pyrophosphate; the active acetaldehyde is subsequently transferred to either pyruvate or α -ketobutyrate.

The most interesting reactions in the two pathways are the conversions that account for the migration of the α -alkyl groups to the carbonyl carbons and the accompanying reduction of the carbonyl to a hydroxy group. The over-all reactions require NADPH₂. A priori these can go by isomerization followed by reduction, or reduction followed by isomerization, or by simultaneous reduction and isomerization. Unfortunately from the point of view of deciding cleanly among these alternatives, both α -keto- β -hydroxy acid reductase and α -hydroxy- β -keto acid reductoisomerase activities have been demonstrated in *E. coli* and *N. crassa* (142). One wonders whether NADPH₂ performs a function in the reductoisomerase similar to that of B₁₂ in the β -methyl aspartate and methylmalonyl coenzyme A isomerase reactions or whether these reactions may be B₁₂-dependent as well. If it were certain that the B₁₂-requiring mutant of Davis & Mingioli (143) was completely blocked, a B₁₂ involvement in these reactions would be improbable since the B₁₂ requirement of the mutant could be met entirely by methionine.

Gerulat & Berg (144) have investigated growth promotion in the rat by D-valine and D-leucine. D-Valine was found to be a very poor replacement for L-valine; even this limited ability was severely antagonized by D-leucine.

These results were interpreted in terms of the relatively poor turnover of D-valine by D-amino acid oxidase and its probable further reduction in the presence of D-leucine. This function of D-amino acid oxidase to act as the only means of inverting the D-configuration was demonstrated by Horowitz *et al.* (145) in *N. crassa*. The inclusion of the D-amino acid oxidase-deficient gene into a methionineless or arginineless organism eliminates their ability to respond to the D-isomer of the respective amino acid.

GLYCINE AND SERINE

In a study of the amount and activity of rat liver phosphoserine phosphatase, Nemer *et al.* (146) have shown that the enzyme can account for the

formation of only 0.15 mM of serine per 100 gm. animal per day. This is only about 8 per cent of the rapidly growing animal's daily requirement of glycine and serine, and hence they have concluded that there must either be alternative synthetic pathways or that other tissues account for the bulk of serine synthesis. In order to substantiate this conclusion, it should be shown that the phosphatase is incapable of an adaptive increase on diets devoid of glycine and serine, since only in the absence of dietary glycine and serine does the inadequate rate become meaningful. However, additional evidence for an alternative pathway has been provided by Hedrick & Sallach (147). They have demonstrated the presence of a hydroxypyruvate-alanine transaminase in human liver, though they note that as yet no metabolic source of hydroxypyruvate is known.

In a detailed study of the phosphoserine phosphatase, Neuhaus & Byrne (148) have found all data compatible with a double displacement mechanism involving a phosphoryl enzyme intermediate. The ability of this enzyme to catalyze an exchange reaction between serine and phosphoserine raised the question as to whether this accounts for the conversion of isotopic serine to ethanolamine. However, though phosphoethanolamine appeared to be the first ethanolamine derivative produced from serine, no evidence could be provided to substantiate a direct decarboxylation of phosphoserine to phosphoethanolamine (149).

When isotopically labelled L- and D-serine were given to the rat, the isotope distribution in glycogen was markedly different (150). The distribution was compatible with a conversion of the L-serine to pyruvate via L-serine dehydrase. The distribution with D-serine could be rationalized in terms of a conversion to 3-phosphoglycerate via hydroxypyruvate and glyceric acid. However, there is no evidence for the occurrence of hydroxypyruvate reductase in mammalian tissues. By use of a different technique, Nadkarni *et al.* (151) have also found a difference in the metabolic fate of D- and L-serine and confirmed the gluconeogenic properties of serine and also glycine.

A metabolic function for D-serine per se in animal systems has only been shown for the earthworm, where it is used for the biosynthesis of the phosphagen lombricine (2-guanidinoethyl-2-amino-2-carboxyethyl hydrogen phosphate) (152 to 156).

Sanadi & Bennett (157) have found that chicken liver mitochondria, osmotically shocked, can carry out the net synthesis of serine from glycine. No cofactor requirements could be demonstrated, indicating that cofactors, as well as the enzymes, remain bound to the particulate complex.

The human disease, hyperoxaluria, provides a confirmation of the metabolic pathway leading from glycine to oxalic acid. Patients with this disease excrete as much as 400 mg. of oxalic acid per day in contrast to the normal value of 20 mg. per day. In both the normal and hyperoxaluric patients, glycine contributes about 40 per cent of the carbon of the oxalic acid (158, 159). However, the glycine metabolic pool in man is large (160), and oxalic acid formation represents a minor part of its metabolism.

THREONINE

The enzyme that converts O-phosphohomoserine to threonine carries out an elimination of phosphate and an isomerization with respect to the OH group. This enzyme has been purified 500-fold by Flavin & Slaughter (161) with no indication that it can be resolved into two activities. However, it was possible to show a stimulation with pyridoxal phosphate. By carrying out the reaction in water labelled with heavy oxygen, it could be shown that cleavage occurs at the C—O bond, and hence this is an elimination reaction rather than a hydrolytic cleavage of the phosphate (162). When D₂O was used as the solvent, deuterium was found in the alpha- and gamma-positions (163). Flavin & Slaughter (164) propose a mechanism for this reaction which involves the formation of an enzyme-bound Schiff base of vinylglycine with pyridoxal phosphate.

In a detailed paper, Elliot (165) has confirmed that *S. aureus* has two means of forming aminoacetone. One pathway involves oxidation and decarboxylation of threonine and the other the condensation of glycine and acetyl coenzyme A with accompanying decarboxylation. Neuberger & Tait (166) have eliminated 1-amino-2-propanol as a possible intermediate in the threonine pathway. An enzyme present in citrated ox plasma can convert aminoacetone to methylglyoxal. Partially purified preparations still contained amine oxidase activity, but, nonetheless, this does represent the first evidence for methylglyoxal formation from a substrate known to occur naturally (167). 2-Aminoacetoacetic acid has been synthesized by Neilands *et al.* (168). Elliot (169) has an improved means of determining aminoacetone and δ -aminolevulinic acid.

LYSINE

One of the most interesting aspects of the biosynthesis of lysine is the existence of two different pathways for its formation, one through 6- and the other through 7-carbon intermediates. The original assignment of the 7-carbon pathway to the bacteria and blue-green algae appears to have been too restrictive. Vogel has presented isotopic evidence for the operation of the diaminopimelate pathway in green algae (170), saprolegniales (171), and higher plants (172). The diaminopimelate pathway has been reviewed by Rhuland (173) and a hypothetical scheme of the biosynthetic sequence presented. This will have to be amended in the light of the finding that aspartic acid is first reduced to aspartic β -semialdehyde before it is utilized for diaminopimelic acid synthesis (174).

The observation by Meadow (175), that penicillin causes a more rapid lysis of a diaminopimelic acid-requiring mutant than diaminopimelic acid deprivation, may be explicable in terms of an incomplete enzymatic block in the mutant. This capability of the diaminopimelic acid auxotroph M-173-25 to synthesize limited amounts of its required nutrients has been shown by Rhuland & Hamilton (176).

The two lysine pathways may not be completely disparate since Sagisaka

& Shimura (177) have indications of a conjugated intermediate which accumulates before α -aminoadipic- δ -semialdehyde in a fractionated enzyme system obtained from *Torula utilis*.

Through use of isotopically labelled pipecolic acid, Rothstein & Greenberg (178) have been able to show its ready conversion to α -aminoadipic and glutaric acids. This places pipecolic acid in the main path of lysine catabolism in the mammal. Evidence of pipecolic acid formation from lysine has been obtained for the turkey (179). In bacteria there appear to be at least two pathways of lysine dissimilation, one resembling the pipecolic pathway (180) and the other degrading lysine via δ -aminovaleric acid (181, 182).

4-Hydroxypipecolic acid has been isolated from the leaves and heartwood of acacia (183), while 5-hydroxypipecolic acid has been detected as a product of δ -hydroxylysine oxidation by rat kidney and liver homogenates (184). Viswanatha & Irreverre (185) have detected hydroxylysine in trypsin. This amino acid is the site of a slow uptake of a second mole of di-isopropyl phosphorofluoridate.

HISTIDINE

The synthesis of the histidine precursor, D-erythroimidazole glycerol phosphate, has been studied in bacterial extracts (186). Ribose-5-phosphate (presumably activated to 5-phosphoribosyl-1-pyrophosphate) condenses with ATP at the N-1 position. The resulting complex is broken down in the presence of glutamine to yield imidazole glycerol phosphate and the purine biosynthetic intermediate, 5-amino-4-imidazolecarboxamide ribotide. This over-all scheme explains satisfactorily the manner in which N-1 and C-2 of the imidazole ring of histidine are derived from N-1 and C-2 of purine.

L-4-Imidazolone-5-propionic acid has played an elusive yet dominant role in the degradative pathway for histidine. The compound has a half life of only 24 min. at pH 7.2, and it was necessary for Brown & Kies (187) to resort to anaerobiosis as well as acidification in order to stabilize the molecule. Aerobically it decomposes to formylisoglutamine. Enzymatically its major fate is conversion to formiminoglutamic acid. The hydrolase that catalyzes this ring-opening cleavage has been partially purified from rat liver (188). However, Brown & Kies (189) have demonstrated that imidazolonepropionic acid may also be oxidized to L-hydantoin-5-propionic acid. The requisite enzyme occurs in the soluble fraction of guinea pig liver. Hydantoin propionic acid is a terminal metabolite and was shown to occur in rat, monkey, and human urine. The chemistry and enzymology associated with the metabolism of imidazolone acetic acid is analogous to that of its higher homologue (190).

Formiminoglutamic acid metabolism appears to be a sensitive indicator of the state of the coenzymes of the 1-carbon pool (191). High-fat diets which deplete the liver of folic acid and B₁₂ cause an enhanced excretion of formiminoglutamic acid, whereas ethionine administration has the opposite effect, in keeping with its reduction in the urocanase activity of the liver (192). Excess methionine also reduces the excretion of formiminoglutamic acid in animals deficient in both folic acid and B₁₂ (193).

The other pathways of histidine catabolism, though they account for only a minor part of the gross metabolism, are of major pharmacological importance. Schayer (194) regards shock as an imbalance of catecholamines and histamine and provides evidence for a regulated interrelationship: a fivefold increase in histidine decarboxylase is induced by the administration of epinephrine to mice.

The enzyme responsible for the methylation of histamine has been purified from guinea pig brain (195); it will not methylate histidine. Brown *et al.* (196) postulate that the methyl histidine present in urine may arise from dietary anserine. Though methylation is regarded as the major fate of histamine in the human (197, 198), Kapeller-Adler (199) could detect only imidazole acetaldehyde as the product of human placental histaminase.

PROLINE

A procedure for obtaining a stable and relatively pure preparation of δ -pyrroline-5-carboxylic acid is described by Strecker (200). An important datum still missing for this compound is the percentage present in the open chain form (glutamic semialdehyde). This has particular bearing on the suggested reversal of the ornithine δ -transaminase to synthesize rather than utilize ornithine (201). The conversion of exogenous glutamic semialdehyde to arginine has been shown by the isotope competition method by Vogel & Kopac (202).

Mitoma & Smith (203) have produced granuloma tissue by carrageenin injection in guinea pigs. The rate of incorporation of isotopically labelled proline was measured in scorbutic and normal animals. It was concluded that under scorbutic conditions incorporation of both proline and hydroxyproline into collagen is decreased but that the conversion of proline to hydroxyproline is unaffected. The injection of proline- U - ^{14}C into rats led to the excretion of pyrrole-2-carboxylic acid, a metabolite of hydroxyproline (204). From the rates of decrease, during six days, in the ^{14}C activity of free hydroxyproline and pyrrole-2-carboxylic acid, Gerber *et al.* postulate the existence in collagen of two metabolically different components characterized by rapid and slow turnover rates. Hydroxyproline-containing peptides have been found in the carrageenin granuloma (205) and in the urine of a patient with rheumatoid arthritis (206). Dekker (207) has detected an enzyme activity in rat liver that can split γ -hydroxyglutamic acid, an intermediate in hydroxyproline catabolism, to yield glyoxylic acid as one of the products.

SULFUR AMINO ACIDS

The observations that methionine could partially replace *p*-aminobenzoic acid in relieving sulfonamide inhibition and that it could entirely meet the needs of a B_{12} auxotroph have suggested a role for these two vitamins in methionine synthesis. This has received direct confirmation through a study of methionine synthesis in washed cell suspensions. Gibson & Woods (208) have investigated the requirements for methionine formation from homocys-

teine in normal and auxotrophic strains of *E. coli*. They have established that these vitamins participate in the final stages of methionine assembly and that serine can efficiently supply the necessary 1-carbon unit. These investigations have been extended to cell-free extracts prepared from acetone-dried organisms. Methionine synthesis in these extracts exhibits a requirement for nicotinamide-adenine dinucleotide, ATP, Mg^{++} , inorganic phosphate, and boiled *E. coli* extract. The *Kochsaff* may be partially replaced by a folic acid derivative obtained from *Clostridium cylindrosporum* (209). It is not replaceable by tetrahydrofolic acid unless the bacteria are grown in the presence of cobalamin (210). When extracts were prepared ultrasonically from a B_{12} auxotroph, an *in vitro* cobalamin requirement could be demonstrated (211). In this system there is some question as to the nature of the endogenous folic acid derivative that participates. Though tetrahydropteroyl glutamate can replace the *Kochsaff* in the presence of cobalamin, tetrahydropteroyl glutamate is unstable in air while the *Kochsaff* factor is not (212).

The methylamide, ethylamide, and analide analogues of cobalamin have been found to be antagonists of cobalamin in methionine synthesis (213). The analide analogue inhibited competitively, which suggests a direct utilization of cobalamin itself. This appears to be borne out by the finding of Tekeyama & Buchanan (214) that B_{12} but not Barker's coenzyme (5,6-dimethyl benzimidazole cobalamin) can reactivate an apoenzyme essential to the synthesis of methionine. Kisliuk (215), who purified the enzyme rather than the apoenzyme, also reports that it contains B_{12} . In addition to the above factors, Hatch *et al.* (216) have revealed a requirement for reduced flavin.

Mudd (217) has shown that S-adenosylmethionine may act as a methyl donor to tyramine in barley extracts; L-methionine, methionine-sulfoxide, and S-methylmethionine were inactive. The barley extracts were also capable of synthesizing S-adenosylmethionine (218). It would appear that methyl group activation and transfer in the plant is analogous to the reactions in animal tissue. The precise mechanism of that activation is still unknown. 3,5'-Cycloadenosine can be added to the list of possible intermediates that have been ruled out (219).

Durell & Steiner (220) have used light scattering to follow an interesting polymerization reaction that occurs with the purified thetin-homocysteine methyltransferase from horse liver. The subunits are held together by disulfide bridges that can be broken by sulfite and thiols. Sloane & Boggiano (221) have provided evidence that betaine and dimethylthetin homocysteine methyltransferases are distinct proteins, though they noted that both enzymes are inhibited by dimethylglycine and thioglycolate. These activities were not depressed in the B_{12} -deficient chick. An S-methylmethionine-homocysteine transmethylase has been detected in rat liver and several microorganisms (222). Bremer & Greenberg (223) report that a water-insoluble aminoethanol derivative can act as a methyl group acceptor in choline synthesis by a rat liver microsome system.

Candida utilis has vacuoles that contain S-adenosylmethionine. Up to 60

μM are accumulated per gm. of cells. No mention is made of the counter-ion which must exist in these vacuoles (224). In yeast, the reduction of methionine sulfoxide to methionine requires the action of three partially purified enzymes (225). One of the enzymes was identified as a relatively non-specific NADPH-linked disulfide reductase. A more specific reductase is to be found in the larvae of the clothes moth. Digestion of wool keratin must provide the larvae with large amounts of cystine, which is then attacked by the reductase (226).

Nakamura & Sato (227) have obtained 14 *Aspergillus nidulans* mutants that require methionine. A class of mutants that gave nutritional evidence of being blocked between thiosulfate and cysteine could be further subdivided into two groups on the basis of heterokaryon tests. One strain so differentiated could not grow on cysteine-S-sulfonate as the sulfur source though the wild type could. This provides further evidence for carbon-sulfur bond formation occurring at the level of thiosulfate.

Carbon-sulfur bond rupture can occur in plants at the sulfite level with β -sulfinylpyruvate as intermediate. A transaminase that operates between cysteinesulfinic acid and α -ketoglutarate is present in oat leaves (228).

Bray *et al.* (229) have implicated a deficiency at the N-acetylation step, rather than an enhanced ability to deacylate the mercapturic acid, as the cause of failure of the guinea pig to excrete large amounts of mercapturic acids (230). Thomson & Young (231) have reported the first example of oxidation of a mercapturic acid to the S-oxide after its formation from bromoethane administered to the rat.

PHENYLALANINE AND TYROSINE

Prephenic acid can be aromatized by both acid- and base-catalyzed mechanisms to yield phenylpyruvic and *p*-hydroxyphenyllactic acids, respectively. The acid-catalyzed reaction has its enzymatic counterpart in prephenic aromatase, which also forms phenylpyruvic acid. However, prephenic dehydrogenase forms *p*-hydroxyphenylpyruvic acid; hence, the enzymatic and base-catalyzed mechanisms that lead to the phenol derivatives would appear to be different (232). The previous report (233) of identical mechanisms was undoubtedly attributable to the presence of lactic dehydrogenase in the enzyme preparations, the reduced form of nicotinamide-adenine dinucleotide, produced by prephenic dehydrogenase, being used in a coupled reaction to reduce the product to *p*-hydroxyphenyllactic acid. *p*-Hydroxyphenylpyruvic acid cannot be an intermediate in the base-catalyzed reaction since it is labile to strong alkali and yields *p*-hydroxybenzaldehyde (234). Shikimic and quinic acids have been implicated as sources of urinary catechol in the rat (235).

An intensive study of the observation that the scorbutic guinea pig excretes *p*-hydroxyphenylpyruvic acid has been made in the hope that it might elucidate the mode of action of ascorbic acid. The major factor responsible for the excretion is the sensitivity of *p*-hydroxyphenylpyruvic oxidase to in-

hibition in the absence of certain reducing agents. Zannoni & LaDu (236) believe the inhibition is attributable to a minor product that is produced as the enzyme acts on its substrate. Ascorbic acid can protect against this inhibition. However, reduced 2,6-dichlorophenol indophenol is 700 times more effective than ascorbic acid. These *in vitro* studies have been extended to the intact animal in which it can be demonstrated that administered *p*-hydroxyphenylpyruvic acid can reduce the oxidase levels in the scorbutic guinea pig but not in the normal animal (237). The dichlorophenol indophenol is also effective in the intact animal. Contributory factors to the tyrosyluria seen in the scorbutic tyrosine-fed guinea pig is the induced increase of tyrosine transaminase, as well as the lower content of *p*-hydroxyphenylpyruvic oxidase (238, 239). The cockroach contains four to eight times as much ascorbic acid per gm. as the guinea pig, so perhaps it is not surprising that the oxidation of tyrosine by dialyzed homogenates of *Blattella conjuncta* can be stimulated by ascorbic acid (240).

The homogentisicase of beef liver has been partially purified by Tokuyama and the ferrous ion requirement confirmed. Exchange with $^{59}\text{Fe}^{++}$ was observed only in the presence of substrate (241). Though the suggestion has been made that the ferrous ion is held by an —SH group, *p*-chloromercuribenzoate does not release the iron (242).

Approaches to the mechanism by kinetics (243) and through the use of $^{18}\text{O}_2$ (244) have been made. Though it was possible to demonstrate the incorporation of one atom of oxygen into the maleyl carboxyl group, the carbonyl oxygen exchanges too rapidly with water to confirm its presumed origin from atmospheric oxygen. The degradation of phenylalanine in cell-free extracts obtained from a vibrio is analogous to the mammalian metabolism since it proceeds to homogentisic acid and this, in turn, can be converted to maleyl-acetoacetic acid (245). Phloretic and *p*-coumaric acid have been identified as urinary metabolites derived from tyrosine (246). However, though *o*-tyrosine sulfate is also a urinary metabolite, it has not been possible to demonstrate its formation *in vitro* (247).

Catecholamines.—Levin *et al.* (248) have solubilized the beef adrenal system that hydroxylates dopamine to norepinephrine. As in the case of the phenylalanine-to-tyrosine conversion, the hydroxylation is coupled to the oxidation of an equimolar amount of a cofactor. In this system, ascorbic acid or certain of its derivatives can serve as cofactor; there is also an unexplained stimulation by fumaric acid. Senoh *et al.* (249) have shown that in the conversion of $\beta,\beta\text{-}^3\text{H}$ -dopamine to norepinephrine the β -hydrogen that is retained does not exchange. This group has also studied the non-enzymatic conversion (250).

Goldstein *et al.* (251) have identified 3-methoxy-4-hydroxyphenylethanol as a minor urinary metabolite of dopamine in the rat. The methylated and unmethylated acetic acid derivatives were the major product. The same types of metabolic fate can still occur after hydroxylation in the beta-position. Inhibition of the O-methylase by intravenous infusion of pyrogallol en-

ables 3,4-dihydroxyphenylglycol to accumulate in the urine (252). This indicates that deamination must in part precede methylation. In the normal animal the glycerol derivative is methylated and appears in the urine as 3-methoxy-4-hydroxyphenylglycol (253, 254). Methylation in the para-position is also possible (254a, 255), as is direct methylation of norepinephrine and epinephrine since normetanephrine and metanephrine have been detected in human urine (256). Apparently there are many alternatives open to dopamine in the course of its metabolism. Kopin (257) offers a suggestion for an isotope dilution technique to quantitate these alternative pathways.

Joly & Nadeau (258) have explained the fact that norepinephrine and epinephrine give similar condensation products with ethylenediamine. Side-chain cleavage during the course of the reaction is assumed to yield identical intermediates for the condensation reaction. Yagi *et al.* (259) have confirmed this interpretation, though they had originally thought it represented evidence for demethylation of epinephrine (260). Yagi & Nagatsu (261) claim that rat liver mitochondria can, in fact, carry out the demethylation of epinephrine to yield norepinephrine.

Aromatic hydroxylation.—In their investigation of a model system for aromatic hydroxylation—a system that involves ascorbic acid, oxygen, and Fe^{++} —Udenfriend *et al.* (262) concluded that a reaction product of hydrogen peroxide and ascorbic acid was the hydroxylating agent and that the mechanism did not involve the OH-free radical since the directed nature of the products precluded the random attack of such a radical (263). In a reinvestigation of this system, Breslow & Lukens (264) have come to rather different conclusions. The attacking species is regarded as the OH radical generated by the interaction of Fe^{++} and H_2O_2 . The function of the ascorbic acid is to reduce Fe^{+++} back to Fe^{++} . In conformity with their theory, they could not replace ascorbic acid with dehydroascorbic acid as originally claimed (262). Acheson & Hazelwood (265) have provided a further substantiation of the free radical mechanism from a careful analysis of the proportions of the isomer products.

Imai & Sato (266) have reported the solubilization of the aromatic hydroxylase system of liver microsomes and the necessity for a lipid-like co-factor.

Aromatic oxidations.—The free radical property of melanins has been verified by Mason *et al.* (267) in several ways. Measurements of electron magnetic resonance indicated an increasing content of unpaired electrons during progressive melanization of the cuticle of the insect *Calliphora erythrocephala* (Meigen). The same observation could be made *in vitro* during tyrosinase-catalyzed formation of melanin from dihydroxyphenylalanine. A physical explanation of this property of melanin is offered by Longuet-Higgins (268).

Roston has observed the ability of $-\text{SH}$ to interact with the intermediates produced through the action of tyrosinase on epinephrine, norepinephrine, and dihydroxyphenylalanine (269, 270). This *in vitro* effect is regarded as a means of regulating melanin production *in vivo*. The ability of

homogentisic acid to form melanin is the basis of ochronosis in the alcaptonuric. The auto-oxidation of homogentisate and the failure of tyrosinase to influence the process were studied by Milch & Schirmer (271). The complexity of the action of tyrosinase is emphasized by an examination of spectral changes that occur with several classes of tyrosine peptides; N-terminal, C-terminal, and interior tyrosine yield different spectral patterns (272). The spectra of polyphenoloxidase products have also been studied by Kodja & Bouchilloux (273).

Miscellaneous observations.—The phenylketonuric individual is subjected to unusually high concentrations of phenylalanine. However, this fails to influence the composition of the hemoglobin he produces (274). His amine metabolism, on the other hand, does appear to be unique. When fed the monoamine oxidase inhibitor, hydrazinephenylpropane, his excretion of phenylethylamine increases 200-fold, whereas in the normal individual the increase is only threefold (275).

Tyrosine and leucine can be incorporated into liver phospholipid, according to Gaby & Silberman (276). However, the difficulties in being sure of compound formation in these lipoamino acid fractions are serious and are discussed by Wren (277). Cameron (278) has studied diiodotyrosine metabolism in goitre and hypothyroidism.

TRYPTOPHAN

Smith & Yanofsky (279) have been able to demonstrate the formation of a 1-(O-carboxyphenylamino)-1-deoxyribulose 5-phosphate in extracts prepared from certain tryptophan auxotrophs. The dephosphorylated compound had previously been identified as an accumulation product in the medium in which this type of auxotroph had grown [Doy & Gibson (280)]. Smith & Yanofsky were also able to demonstrate the further conversion of their compound to indole-3-glycerolphosphate. As yet no intermediate steps have been recognized either in the formation of the Amadori-type compound from anthranilic acid and 5-phosphoribosyl-1-pyrophosphate or in its conversion to indole-3-glycerolphosphate, which involves a decarboxylation as well as a ring closure. Reduction of the Amadori compound would give a ribotyl side-chain. One wonders if the ribotyl residue of flavin mononucleotide originates in this way.

In a study of the tryptophanase from *E. coli*, Turner & Happold (281) noted that, in contrast to glutathione, the protective effect of cysteine against chloroacetophenone was obscured by coenzyme removal. Ascorbic acid was inhibitory under all conditions. The action of tryptophanase on a number of methyl and chloro derivatives of tryptophan has also been examined (282). An attempt was made to correlate changes in affinity constants and first-order velocity constants with the electronic properties of the substituents. While K_m changes could be correlated, K_s changes could not.

In the pseudomonad degradation of kynurenic acid, Behrman & Tanaka (283) have noted that 7,8-dihydroxykynurenic acid will give the same inter-

mediary products as kynurenic acid itself. One of these intermediates is postulated to be β -(2,6-dicarboxy-4-hydroxypyridyl-3) acrylic acid. An end product is glutamic acid. These same observations have been made by Hayaishi *et al.* (284). The mammalian kynurenine transaminase can combine with the sulfate esters of diethylstilbestrol, estradiol, and estrone. This combination is in competition with pyridoxal phosphate and results in an inhibition of enzyme activity. On the other hand, it also protects the enzyme against the action of proteolytic enzymes (285).

Serotonin.—There is still some uncertainty as to whether the decarboxylations of 5-hydroxytryptophan and dihydroxyphenylalanine are carried out by the same or different enzymes. In the original description of a purified 5-hydroxytryptophan decarboxylase, it was noted that the ratio of these two activities changed during the purification (286). However, this has now been attributed to a pyridoxal effect (287), and a variety of inhibitors, supposedly specific for dihydroxyphenylalanine decarboxylase, affect the 5-hydroxytryptophan enzyme (288). Another instance of common identity is the transaminase for 5-hydroxytryptophan (289), which was previously detected as a tryptophan transaminase. The pH optimum, adaptive response to tryptophan, and thermolability of the enzyme are the same for both substrates.

Rat liver can form the sulfate when incubated with high concentrations of serotonin. Ordinarily this derivative does not appear as a urinary metabolite. However, if iproniazid is administered to block monoamine oxidase, then sufficient serotonin sulfate forms to spill over into the urine (290). Serotonin can also be attacked by serum oxidase to yield, ultimately, brown pigments. Eriksen *et al.* (291) have evidence of a dimer as an intermediate in this oxidation. The same intermediate can be obtained using silver as the oxidizing agent. An enzyme similar to the serum oxidase, but less specific, has been detected in the gill plates of the mollusc *Mytilus edulis* (292). The enzyme can be inhibited by cyanide. Possible model systems for this type of reaction include the vanadium-catalyzed oxidation of 5-hydroxyindoles (293) and the observation that in denaturing oxyhemoglobin an approximately equivalent amount of serotonin is oxidized (294). This latter finding is presumed to account for the ability of washed erythrocytes to destroy serotonin.

Udenfriend *et al.* (295) have determined the content of the pharmacologically active amines in common fruits and vegetables. It is fortunate that orally administered amines are inactive since the amine content is rather high in some instances.

ATP has been found in association with serotonin in cytoplasmic particles in the small intestine of the dog. The ATP:serotonin ratio is 1:3 (296) which is about right if the ATP functions to neutralize the positive charges on the serotonin. The finding that serotonin is able to increase lactic acid production in the liver fluke is referred to its ability to facilitate the synthesis of cyclic adenosine monophosphate and hence to activate phosphorylase (297). Serotonin appears to play the same role in this system as epinephrine in mammalian liver and ACTH in the adrenals.

Melatonin.—A detailed paper by Lerner *et al.* (298) has appeared on the isolation of melatonin (N-acetyl-5-methoxytryptamine) from bovine pineal glands. This interesting compound lightens the color of frog skin by causing an aggregation of melanin particles. It is effective at concentrations as low as 10^{-12} gm. per ml. The pineal gland also contains unusual amounts of 5-methoxyindole-3-acetic acid (299). However, this compound is not biologically active. These compounds represent the first-reported instances of the presence of 5-methoxyindole derivatives in tissue. Lerner & Case (300) have pointed this out as an example of increase of biologic potency through O-methylation, which is the opposite of the situation in the catecholamines. The enzyme responsible for the methylation has been partially purified from beef pineal gland (301). The absence of the enzyme from liver and kidney and its lack of a Mg^{++} requirement clearly distinguish it from other O-methyl transferases. Serotonin is methylated at only one-tenth the rate of N-acetyl serotonin, which suggests that acetylation precedes methylation in melatonin formation.

Though 5-methoxyindole-3-acetic acid represents the major metabolic fate of 5-methoxytryptamine (302), acetylation diverts from this pathway, and hydroxylation becomes pre-eminent (303). The sulfate and glucuronide conjugates of N-acetyl-5-methoxy-6-hydroxytryptamine have been demonstrated to be the principal urinary metabolites of melatonin (304).

Urinary metabolites.—Isotope administered as quinaldic acid does not appear in simultaneously fed kynurenic acid, thus indicating that quinaldic acid formation from kynurenic acid is irreversible (305). When quinaldic acid is administered to the rat, it is largely eliminated as such, whereas xanthurenic acid is converted to the sulfate and glucuronide as well (306). Indoyl-3-carboxylic acid is also obtained as the glucuronide (307). The red fluorescent substance in the urine of pyridoxine-deficient rats has been identified as 8-hydroxyquinaldic acid (308). In rats given tryptamine, indole-3-acetic acid is the only end product. However, in rats given the aldehyde oxidase inhibitor, Disulfiram, tryptophol (indolyethyl alcohol) is also obtained (309). This is another example of the ability of a drug to direct the metabolism of a pharmacologically important substance.

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THE STRUCTURE OF PROTEINS^{1,2,3}

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In a year which has been noteworthy for substantial achievements in protein chemistry two groups of papers, each the culmination of many years of investigation, may be said to stand out. In both instances, the detailed structural information which has accrued stimulates the hope that the structure and function of the proteins in question may soon be definitively reconciled. It is therefore proposed to focus attention on these selected topics rather than attempt a survey of the entire field of protein structure, which would inevitably take the form of a catalogue of references.

Firstly, Kendrew and his co-workers (1) have extended the resolution of data from their x-ray diffraction patterns of myoglobin to the 2 Å level, so that for the first time the helical arrangement in the polypeptide chain of a protein can be unequivocally demonstrated. At the same time, Perutz *et al.* (2) have published the x-ray structure of haemoglobin at a resolution of 5.5 Å, and, because of its very great similarity to the myoglobin structure, they have been able to infer considerably more than the actual resolution allows. From these x-ray diffraction patterns one may deduce detailed information on the secondary and tertiary structure of the proteins in the crystalline form. At the other end of the scale is the work of Moore and Stein and their associates (3, 4, 5), who have completed for the first time the determination of the primary sequence of an enzyme, ribonuclease, and have also established the location of the four disulphide bonds in the molecule.

With this fundamental information on the three proteins as a background, it is our purpose to bring together much of the other work that has dealt, during the period under review, with their physical chemical properties especially those pertinent to the secondary and tertiary structures of the proteins in solution. It is this aspect, in addition to a knowledge of their primary sequence and crystalline structure, which will allow the biochemist to interpret reactions involving proteins in living systems. Consideration of the tertiary structure of proteins leads to a further main topic about which there has recently been much discussion: the use of differential spectroscopic methods for the investigation of intramolecular bonding and conformational changes in proteins.

¹ The survey of the literature pertaining to this review was concluded in February, 1961.

² The following abbreviations are used: HbA for the main haemoglobin in normal adult blood; HbC for haemoglobin C; HbE for haemoglobin E; HbF for foetal haemoglobin; HbG for haemoglobin G; HbH for haemoglobin H; HbI for haemoglobin I; HbS for sickle-cell haemoglobin.

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MYOGLOBIN AND HAEMOGLOBIN

Molecular conformation and the haem-protein linkage.—The first report on the three-dimensional structure of crystalline sperm whale myoglobin (6, 7) described a three-dimensional Fourier synthesis of the electron density in the unit cell at a resolution of 6 Å; isomorphous replacement was used to determine the phase angles of the diffracted rays. At this resolution the general shape of the molecule was clearly defined, and most of the course of the polypeptide chain could be followed, although its direction was somewhat ambiguous when it turned through a large angle. The haem group could be seen as a disk of high electron density on the surface of the molecule. Since then, Scouloudi (8) has used essentially similar techniques to obtain a two-dimensional Fourier projection of the unit cell of seal myoglobin; from this she was able to show that the tertiary structures of these two myoglobins are almost identical despite quite marked differences in their amino acid compositions.

Kendrew *et al.* (1) have now published the results obtained for sperm whale myoglobin on extending the resolution to 2 Å. This level of resolution is not quite sufficient to resolve neighboring covalent atoms. However, the polypeptide chain itself, segments of which appeared at the 6 Å level as solid rods with approximately circular cross section, showed up at the higher resolution as hollow cylindrical tubes with regions of high electron density which projected at intervals along the core and corresponded to the amino acid side chains. A detailed analysis of the polypeptide chain showed that the observed electron density follows the configuration of the α -helix of Pauling & Corey, with the side chains emerging at intervals of 100° around the periphery of the helix and at axial displacements of 1.5 Å. One of the more interesting features of the synthesis is that it demonstrates that the α -helix, at least for this protein, is right-handed in sense. It was obvious, even at the 6 Å level of resolution, that such a convoluted polypeptide chain could not be in the form of an α -helix throughout its whole length, since the helix must be disrupted when the chain turns a corner. From the synthesis at the higher level of resolution, Kendrew *et al.* have been able to calculate that 65 to 72 per cent of the polypeptide chain is in the α -helical configuration. It would, of course, be very interesting to be able to compare this value for the helical content in the crystalline form with estimates for proteins in solution obtained from optical rotatory dispersion data, since the latter data are currently interpreted on an essentially empirical basis. Unfortunately, measurements of optical rotation for myoglobin are complicated by the spectral absorption bands associated with the haem group, which give rise to Cotton effects in the rotatory dispersion curves (8a); however, some preliminary results (9) with data which have been corrected for these anomalous effects suggest that the helical content of myoglobin in solution is very similar to that in the crystalline solid. This agreement is sufficient to identify the more commonly occurring α -helical form in synthetic polypeptides with the right-handed conformation.

Estimates of the helical content of myoglobin in solution have been made by Benson & Linderstrøm-Lang (10, 11) from data on the deuterium-hydrogen

exchange behavior of the protein. At pH 7 and 0°C. the estimated helical content was 51 per cent, the highest for any of the conditions investigated.⁴ This is considerably lower than the value of Kendrew *et al.* (1) for the crystalline form. It is difficult to decide whether this reflects an actual difference in configuration between the two states. Scheraga and co-workers have shown for a synthetic polypeptide (12) and for ribonuclease (13) that, under conditions in which the stability between the helix and coil forms is marginal, it can no longer be assumed that replacement of hydrogen by deuterium does not affect the helical content of the molecule. Benson (11) has noted for myoglobin that the value for the number of hydrogens exchanged instantaneously was consistently higher when H in water was exchanged for D in protein than when D in water was exchanged for H in protein. This, together with the sensitivity of the estimated helical content to pH and temperature, suggests that perhaps the exchange of deuterium for hydrogen does in fact affect the helical content of the molecule.

Perutz *et al.* (2) have constructed a three-dimensional Fourier synthesis for horse oxyhaemoglobin at a resolution of 5.5 Å. Although this level of resolution is too low to provide the detail of primary structure evident in the myoglobin work, the convolutions of the four polypeptide chains that make up the molecule and the positions of the four haem groups are clearly delineated. The four chains are made up of two apparently identical pairs. In spite of some differences, the chains exhibit a gross conformational similarity to each other and also to the myoglobin polypeptide chain. In the haemoglobin molecule there are extensive regions of contact between the two sets of chains, which are paired in such a way as to give rise to a roughly spheroidal molecule with a hole through the center.

The haem groups in myoglobin and haemoglobin emerge from pockets in the surface of the protein and make contact with the polypeptide chain in at least four different points. Two long-debated questions on important features of the molecular structure are thus at once resolved: firstly, the hypothesis (14 to 17) that the haem-groups are buried in crevices in the protein structure is invalidated, so that indirect argument from the reactivity of the haem-iron toward various reagents (18) becomes redundant; secondly, the distances between the haem-groups in the horse haemoglobin molecule preclude any direct interaction, so that the haem-haem interactions evinced in the oxygenation process must in some way involve the protein [cf. Williams (19)].

The answer to another important and vexing problem (20, 21, 22) in haemoglobin chemistry also emerges directly from the crystallographic data: the iron-linked group in the protein has been identified, categorically in the case of myoglobin and tentatively in haemoglobin, as a histidyl imidazole ring. In metmyoglobin a water molecule in the sixth iron co-ordination position, distal to the imidazole, is also recorded.

It appears to be established that interactions of the propionyl side chains

⁴ Such estimates are very dependent on the pH and temperature at which measurements are made.

of the haems with the protein also contribute toward the haem-protein linkage. This is to be seen from the stability of "artificial" haemoglobins prepared by the combination of modified haemins (23, 24), in which the propionyl side-chains are missing, with globin. Moreover, Teale (25) and Rossi-Fanelli, Antonini & Caputo (26) have described the formation of a protoporphyrin-globin which is electrophoretically and ultracentrifugally identical with native haemoglobin and which has a spectrum with sharper and intenser bands than the free porphyrin. The binding efficiency of this protoporphyrin was found (25) to be no less than one-third of that for ferriprotoporphyrin (haem), and, since native haemoglobin itself binds no protoporphyrin or haemin, the binding sites must be the same. It may be noted that salt linkages between polar groups in the protein and the propionyl side chains of the haem have been proposed for other haem-proteins (27, 28).

The preparation of globin by acid fission of haem from haemoglobin has been studied by Rossi-Fanelli and co-workers (29, 30), and a modification of this technique has been devised by Teale (31). The globin exists in solution in dissociation equilibrium (mol. wt. = 41,000). It may be said to be a native protein in that reconstituted haemoglobin prepared from it is identical with the native pigment, not only in its physicochemical characteristics (size, shape, spectrum, etc.), but also in its oxygen-binding behavior, both in respect of haem-haem interaction and Bohr effect (30). Rossi-Fanelli & Antonini (32) have also studied the oxygen uptake of haemoglobin prepared from globin and deuterohaemin (in which the vinyl side chains of the porphyrin are absent) and have noted only a small decrease in haem-haem interaction, the Bohr effect being unchanged. They therefore concluded that the vinyl groups contribute to the haem-haem interaction and that their absence is accompanied by a loosening of the tertiary structure of the molecule as a whole. The preparation and spectra of a series of artificial haemoglobins (23, 33) and myoglobins (24) of this kind have been described, and the kinetics of carbon monoxide binding by meso- and deuterohaemoglobin in comparison with protohaemoglobin have been investigated by Smith & Gibson (33) and Antonini & Gibson (34).

A study of the kinetics of recombination of haem with globin indicates that the rate is governed essentially by a preliminary process involving the haem. This is most likely to comprise a disruption of the micellar structures, in which haem is known (37) to exist in solution. Thus, carboxyhaem reacted rapidly and formed, reversibly, an intermediate complex which was converted by a first-order mechanism to carboxyhaemoglobin (35, 36). Accordingly, it was suggested that carboxyhaem is monomeric in solution.⁵

Rossi-Fanelli & Antonini (41) have made the interesting observation

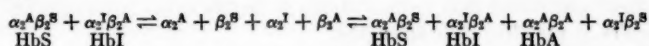
⁵ The depolymerization of haemin in aqueous solution by the addition of ethylene glycol has been observed by Smith (38). The spectroscopic changes accompanying this process are very similar to those described by Keilin (39, 40) on addition of nitrogenous bases to haem solutions. These observations lend support to the suggestion (26) that Keilin's spectra arise from changes in the aggregation state of the haem rather than from binding of the bases.

that haem interchanges in neutral solution between horse metmyoglobin and the apoprotein from the myoglobin of *Aplysia* (a marine mollusc), the metmyoglobin of which is spectroscopically distinguishable from that of the horse. Similar exchange was found to occur between deuteromethaemoglobin and free protohaemin. It is thus clear that a dissociation equilibrium between protein and prosthetic group obtains in haemoglobin and myoglobin at neutral pH and room temperature.

Dissociation and recombination of the haemoglobin chains.—It is known that at both low (42) and high (43) pH human carboxyhaemoglobin is dissociated into subunits, each consisting of two polypeptide chains. The individual chains are designated α and β , so that the whole haemoglobin molecule may be designated as $\alpha\beta_2$. The alternatives then arise of symmetrical or asymmetrical dissociation, that is to say, with the formation of identical $\alpha\beta$ units or of α_2 and β_2 fragments. If two different haemoglobins are allowed to dissociate by exposure to the appropriate pH and are then recombined by neutralization, the possibility arises that new hybrid species may be formed. This process is referred to as hybridization.

Singer & Itano (44) showed that when mixtures of normal adult human haemoglobin (HbA) with an abnormal variety, such as HbS (sickle-cell haemoglobin) or HbC (both now known to be anomalous in the β -chain), were dissociated at low pH and allowed to recombine no new species were formed. If, however, one of the species involved in the hybridization was labelled, either radioactively or in terms of the oxidation state of the haem groups (i.e., was present as the methaemoglobin), interchange of half-molecules was demonstrable.⁶ This evidence was taken to indicate that the dissociation is in fact asymmetrical, that is, into α_2 and β_2 .

If now two haemoglobins, one aberrant in the α - the other in the β -chains, be dissociated and recombined, new species should be formed according to the following scheme, in which HbA is represented by $\alpha_1^A\beta_1^A$, HbS by $\alpha_1^A\beta_2^S$ (abnormal in the β -chain), and HbI by $\alpha_2^I\beta_1^A$ (abnormal in the α -chain):



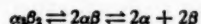
The last species is thus a new and doubly abnormal form. Mixtures involving HbA, HbI, and HbS or HbC were investigated in this manner by Itano & Robinson (46), and free-boundary electrophoresis patterns after hybridization revealed changes consistent with the scheme outlined. It was also found that two new (hybrid) species were formed when HbA was hybridized with canine haemoglobin. Robinson & Itano (47) repeated these experiments in alkaline solution and found that hybridization occurred in the same way.

* An objection presents itself to both methods of labelling: haem interchange can occur independently of dissociation, as already described, and will indeed be favored under acid conditions. Similarly, it is known (45) that the radioactive-labelling technique, whereby the protein is incubated with C^{14} -labelled glycine, leads to incorporation of a considerable portion of the radioactivity in the haem.

They concluded that the dissociation at high pH was also asymmetrical.

Although the over-all results of these hybridization experiments can be explained simply in terms of asymmetrical dissociation and reassociation, it would seem that the dissociation process is more complex than was previously supposed. Charlwood, Gratzer & Beaven (48) have pointed out an inconsistency between the slow alkaline denaturation rate for human foetal haemoglobin (HbF) and the concept of asymmetrical dissociation. Under standard conditions (pH 12.8) the time for half-reaction was some 100 times greater for HbF than for HbA. At this pH, HbF was shown to be fully dissociated. Since the α -chains of the two proteins are thought to be identical (49, 50), the rate curve for HbF should be made up of two sections, one referable to a labile, the other to a resistant species. This is not, in fact, observed. It follows therefore that, unless the α -chains of HbA and HbF are not in fact identical, the dissociation is not a simple asymmetric process.

Vinograd & Hutchinson (51) have suggested that the results of hybridization experiments may be explained in terms of symmetrical dissociation if a further equilibrium between the half-molecules and quarter-molecules be postulated:



This equilibrium may be overwhelmingly in favor of the half molecules as long as free exchange of individual chains is permitted. In order to explain the hybridization results, it must then be further supposed that there can be no recombination between unlike half molecules ($\alpha_A\beta_A$ and $\alpha_A\beta_S$) or quarter molecules (β_A and β_S). A specific α - α and β - β interaction in haemoglobin is thus implied. Support exists for the dissociation of haemoglobin into quarter molecules. Reichmann & Colvin (52) and Kurihara & Shibata (53) have presented evidence for the occurrence of such species in horse haemoglobin under denaturing conditions, and Hasseroth & Vinograd (43) noted a further decrease beyond the half-molecule value in the sedimentation constant of HbA when the pH was raised above about 11.2. Rossi-Fanelli, Antonini & Caputo (26) have also found that human globin is largely dissociated into quarter molecules at very low ionic strengths.

Whatever the mechanism, hybridization experiments have been extensively used for the characterization of human haemoglobin variants. Thus, Vinograd, Hutchinson & Schroeder (54) showed that only the α -chains of HbA and HbS are interchangeable. The α - and β -chains are defined in terms of their N-terminal sequences, Val·Leu- and Val·His·Leu-, respectively, so that incubation with ^{14}C -leucine makes it possible to label the N-terminal peptides specifically. By separating the hybridized haemoglobins, preparing dinitrophenyl-peptides from both components, and measuring their activity, interchange of only α -chains could be established. Jones, Schroeder & Vinograd (49) also found that the α -chains, though not the β -chains, of HbA and HbF could be interchanged, and Jones *et al.* (55) demonstrated by hybridization with HbS, that the variant HbH had the structure β_4^A , that is to say, contained no α -chains.

Benhamou *et al.* (56) have shown that human haemoglobin will dissociate into half molecules at neutral pH in media of high ionic strength: low protein concentration favored this dissociation. Rossi-Fanelli, Antonini & Caputo (57, 57a) examined the oxygen-binding curve of haemoglobin at high salt concentration, where the pigment must be present in dissociated form (57b), and obtained the remarkable result that the Hill constant n , which is regarded (20) as a measure of the haem-haem interaction, is perceptibly increased rather than decreased.

Denaturation of haemoglobin.—The acid denaturation of horse methaemoglobin has been exhaustively studied by Beychok & Steinhardt (58), who were able to refute a previous suggestion by Tanford (59) that the large increase in acid-binding capacity accompanying the denaturation is attributable only to an increased electrostatic interaction parameter, w . Beychok & Steinhardt (60) showed that, irrespective of any change in w , 22 new dissociating groups became exposed; these were subsequently identified as histidyls. At 0° and 25° different products were formed and were characterized by different w values and intrinsic viscosities. In formate buffer the two forms were interconvertible. It appears from the data that the 25° product has the more compact structure. It is of interest that the denaturation process seems to be wholly reversible (61). The formation of different products during acid denaturation at high and low temperature was confirmed in a kinetic and calorimetric study by Forrest & Sturtevant (62), who also proposed a molecular expansion at low temperature.

Amino acid sequences.—The now familiar "fingerprinting" technique has been used to establish aberrations in a number of abnormal haemoglobins. A specific amino acid substitution, in each case in the β -chain, has been established for HbS (63, 64), HbC (64, 65), HbG (66) [all of which are aberrant in residues located near the N-terminus of the chain (64, 64a, 66)], and HbE (67). An α -chain defect in HbI has also been described (67a).

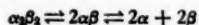
It is possible to separate the α - and β -chains of denatured human globins by column chromatography in a urea gradient at low pH (68, 69) and by countercurrent distribution (70). The fingerprinting technique has been applied to the separated chains, which can be identified by their N-terminal sequences, and a number of interesting results have been reported. Several tryptic peptides from the human α -chain have been analyzed by Hill & Konigsberg (71), and Braunitzer and his co-workers (72, 73, 73a), in particular, have determined the amino acid sequences of large portions of both chains; indeed, there seems good reason to hope that a complete analysis will be achieved in due course. It is already apparent that the α - and β -chains are in large measure homologous: many sequences appear in closely corresponding positions in both chains (73). Taken together with recent reports (74, 75) on the "fingerprint" similarity of the haemoglobins of other species, particularly those of primates, to HbA, this work invites speculation about the evolution of the haemoglobin molecule (76, 90).

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peptide chains are derived from a primitive single α -chain. With the possibility of aggregation of the chains, the advantages of haem-haem interaction are realized. The human α -chain is common to HbA, HbF, and HbA₂, and the N-terminal sequence which defines it is found also in various animals. The α -chain is thus regarded to be essentially invariant (in large measure necessarily so since it has to combine with three different types of β -chains associated with the above haemoglobins in man), and the precursor, by a series of mutations, of the other chains. Repeated gene duplication and translocation are thus involved in the evolutionary process. Ingram's argument is extensively documented and reference should be made to his paper (90).

In connection with the crystallographic analysis by Perutz and co-workers, discussed above, structural data on horse haemoglobin are of particular significance. The polypeptide chains have been separated and are characterized by Val·Leu- and Val·Glu·Leu-as N-terminal sequences (77, 78); amino acid analyses of these chains have been carried out (79). Horse haemoglobin is, however, heterogeneous, there being two major components. Perutz *et al.* (80) find that these differ only in a single trypsin-resistant peptide; it may therefore be inferred that they have one polypeptide chain in common. Similar conclusions have been derived for the haemoglobins of other species (76).

The two reactive sulphhydryl groups of the horse haemoglobin molecule lie in the "black" polypeptide chains (2) at points near the haem-protein link. This is of relevance in considering haem-haem interactions (81). For human haemoglobin, where only two of the six sulphhydryl groups are accessible to specific reagents (82),⁷ Riggs & Wells (83a) showed by coupling ¹⁴C-labelled N-ethylmaleimide and separating the polypeptide chains, that the reactive groups are in the β -chain. Of particular interest are the recent findings of Benesch & Benesch (84). It had been observed (85) that the Bohr effect is suppressed when the active sulphhydryl groups are coupled with N-ethylmaleimide, and this was taken by Riggs (85) to indicate that the oxygenation-linked acid groups of haemoglobin were indeed the sulphhydryl residues. Benesch & Benesch have now shown, however, that the suppression of the Bohr effect arises from a secondary reaction in which a histidyl residue is apparently acetylated by the imide grouping of the sulphhydryl-mounted N-ethylmaleimide ring. Moreover, when this reagent is coupled with reduced haemoglobin the Bohr effect remains intact. This provides tangible evidence in favor of the steric changes which have been postulated to occur during the oxygenation process (19).

A factor which has to be taken into consideration in work on haemoglobin and myoglobin is the evident heterogeneity of these proteins. Rat and seal myoglobin are found to have, respectively, three and five components (86, 87), whereas in human haemoglobin varying numbers of minor components have been reported, especially by ion-exchange chromatography (88, 89). Only one of these has been established as an independently synthesized

⁷ Cf. also data for HbF and HbS (83).

entity differing in β -chain composition from HbA (90, 91). The nature of the other components is uncertain (92), although it is possible that they may be successive physiological degradation products, differing perhaps only in terms of secondary or tertiary structure.

RIBONUCLEASE

Three recent papers (3, 4, 5) described the outcome of several years work by Stein and Moore and their associates in elucidating the complete primary structure of ribonuclease and locating the four intrachain disulphide linkages (cystine residues) in the molecule. The most troublesome aspect of the closing stages of the work was the selection of a method of hydrolysis to give cystine-containing peptides so that the positions of the —S—S—bridges could be determined. Under conditions similar to those successfully used by Ryle *et al.* (93) in their work on insulin, it was found that disulphide interchange [Ryle & Sanger (94)] occurred in ribonuclease, so that the positions of the cystine linkages could not be unequivocally determined. As a result of control experiments with model disulphides, the method of hydrolysis chosen involved cleavage of the molecule with pepsin at pH 2.

Tertiary structure from models.—The elucidation of the primary structure for this molecule has stimulated the construction of three-dimensional models, in which it was hoped that a correlation could be achieved between the primary structure and the enzymatic and physical chemical properties of the protein. Three such models have been proposed (95, 97), each differing considerably from the others. In each case the underlying assumption is made that the structure contains as much right-handed helix as possible. The justification for such an assumption is questionable, however, since optical rotation (98), infrared spectra (99), and deuterium exchange (100) indicate that less than half of the residues are in the helical configuration. On the other hand, when dissolved in 2-chloroethanol, in which the protein might be expected to have its maximum helical content, the helical content derived from optical rotatory measurements rises to *ca.* 70 per cent (98); this agrees closely with the calculated value for the models of maximum helical content. Thus, although such models may give some indication of the conformation of the molecule under certain specialized conditions, they probably do not give a good representation of the situation in aqueous solution.

Scheraga's model (95) is based on interpretations of a large amount of physical chemical data in terms of internal hydrogen-bonding and side-chain interactions within the molecule. This model has been criticized (101) in that it does not fit the molecular dimensions deduced from x-ray studies of ribonuclease crystals (102). A very different model has been presented by Parks (97). This is based essentially on current concepts of the enzyme-substrate interaction. It seems likely that many different models could satisfy most of the known properties of the molecule. In the absence of crystallographic data or even definitive information on helical content, it seems questionable whether model building can at this stage make any useful contributions to our knowledge of the enzyme.

Ribonuclease as an enzyme.—In this section we will only deal with chemical and physical chemical studies of the protein insofar as they throw light on the structural requirements for enzymatic activity, rather than on the more general question of the nature of enzyme action itself.

It has been known for some years that crystalline bovine ribonuclease contains two major fractions, A and B (which have almost equal enzymatic activity), as well as at least two minor components (103). Several active components have also been separated chromatographically from ribonucleases of human urine and sperm (99) and of mouse pancreas (100). Aquist & Anfinsen (103) found at least eight biologically active fractions in sheep ribonuclease; four were similar to the bovine fractions in that they were adsorbed under appropriate conditions on Amberlite IRC-50 ion-exchange columns, and four additional components were unadsorbed. The latter were converted irreversibly into the former merely by passage through the column. Anfinsen (105) has turned the species differences to good account; he contends that regions of the molecule in the vicinity of the residues which differ from one species to another are unlikely to be involved in the active site of the enzyme. He is examining the differences in amino acid sequence between a number of species in order to narrow down the region of the molecule involved in the active site. A similar approach has been used by Brown, Tacey & Anfinsen (106) to determine the nature of the antigenic site in some immune bodies derived in experiments in which one species of ribonuclease was cross-reacted with the antibody to another species. If there were a marked difference in reactivity, the region of the polypeptide chain in which there are species differences might be correlated with the antigenic site. So far these differences in reactivity have not been found.

Considerable interest has recently been focused on inactivation of ribonuclease by chemical modification of specific residues. It has been argued (107) that, if certain residues which are far apart in the primary sequence are essential for activity, deductions could be made about the tertiary structure of the molecule, since it would have to be folded in such a way as to bring the essential residues into some juxtaposition. However, this approach could be somewhat misleading, since the tertiary structure could well be broken down by specific chemical modification of a residue which is not in itself necessary for enzymatic activity and is far removed from the "active center" of the molecule. It seems difficult to decide in such cases whether the loss in activity on chemical modification is caused by a change in the active center or by a less specific disruption of secondary or tertiary structure. Gundlach, Stein & Moore (107) have studied the inactivation of ribonuclease by reaction with iodoacetic acid over a wide range of pH. They have shown that in acid (pH 2.8) the inactivation is predominantly attributable to reaction of the iodoacetic acid with methionine residues; in alkaline solution (pH 8.5) reaction is with both methionine and lysine; and at an intermediate acidity (pH 5.5) reaction is almost exclusively with one of the four histidine residues. Barnard & Stein (108, 109) have made a similar study but have confined their attention to the inactivation resulting from the specific interaction between bro-

moacetic acid and one of the histidine residues in neutral solution. By coupling with ^{14}C -labelled bromoacetic acid, followed by partial acid hydrolysis and chromatographic separation of the fragments, they showed that the histidine residue involved is the one nearest the C-terminal end of the polypeptide chain. This in itself is interesting, since Richards (110) had previously shown that photo-oxidation of the histidine residue nearest the N-terminal end of the molecule destroyed activity. In this case we may be justified in assuming that the two ends of the molecule are in spatial proximity but not necessarily involved in the active center. More recently Barnard & Stein (109) have studied the bromoacetate-ribonuclease reaction in 8 *M* urea. It will be recalled that, although ribonuclease is extensively unfolded in 8 *M* urea solution, its activity is not lost, since the addition of polyanions, such as the substrate, is sufficient to reform the molecule (104). If the bromoacetic acid reagent is then added in the presence of 8 *M* urea at pH 5.3, no activity remains after dialyzing away the urea, and one histidine residue is found to have reacted. Moreover, if the enzyme is exposed to higher pH values (~ 10.5) in 8 *M* urea, it is not only inactivated but will not then react with bromoacetic acid at pH 5.3. This suggests that there is a specific tertiary structural arrangement which both confers enzymatic activity on the protein and at the same time activates a particular histidine residue so that it alone of the four in the molecule can react with bromo- or iodoacetic acid. Irreversible disruption of the tertiary arrangement by urea at pH 10.5 results in the loss of both of these properties.

The approach of Richards and associates (111, 112, 113) is doing much to illuminate the tertiary structure of the molecule and distinguish residues involved in the catalytic site. Richards (111), using the enzyme subtilisin, has split a single peptide bond in the molecule at a point 20 residues removed from the N-terminal end. If the peptide and protein moieties are separated, neither is enzymically active, but if they are subsequently mixed together full activity is regained in spite of the broken peptide bond. This indicates that there must be a very specific interaction between the peptide and the protein portion, which is intimately associated with the enzymatic function of the molecule. Having characterized the process of splitting and recombination in detail, Vithayathil & Richards (113) studied the effects of chemically modifying various side chains in the 20-residue peptide. In such a relatively small unit, modifications are easier to determine and the situation is not complicated by possible changes in secondary structure or denaturation. They first considered the effects of chemically modifying the amino groups on the peptide—one αNH_2 and two lysine ϵNH_2 groups. On deamination with nitrous acid or guanidation with *O*-methylisourea, complete activity was regained on mixing the peptide with the protein moiety. No loss in activity occurred if the αNH_2 group was acetylated, but if one or both of the two ϵNH_2 groups were acetylated only about 40 per cent of the activity was restored. Since not all the activity was lost, the acetylation may have affected not so much the active center itself as the tertiary structure of the polypeptide-protein complex.

The relationship between the cystine cross-links in ribonuclease and its enzymatic activity has received detailed attention. In particular, White (114, 115) has shown that the four cystine bridges in ribonuclease can be completely reduced to the sulphydryl form to give a product with no enzymatic activity; reoxidation of the half-cystines by air under appropriate conditions then leads to a fully active regenerated protein. The main requirement is that the concentration of the protein during reoxidation not exceed 0.1 per cent, presumably to minimize random intramolecular cross-links. Even under optimal conditions, White found that these were not entirely eliminated, and only about 70 per cent of the reoxidized protein was soluble; however, the soluble material had about 90 per cent of the biological activity of the native protein. Furthermore, the viscosity, optical rotation, and ultraviolet spectrum, which for the reduced ribonuclease were all characteristic of a denatured protein, indicated that the reoxidized product was essentially native. These results are extremely important since they demonstrate that even drastic alteration of both the secondary and tertiary structure of the enzyme is reversible under certain conditions. Bello (116) has recently reported that, although the reduced and reoxidized protein crystallizes very easily, its crystal form is quite different from native ribonuclease crystallized under the same conditions. However, as Bello points out, this difference may not be structurally significant, since the native material contained the chromatographically separable ribonucleases A and B, whereas the regenerated sample consisted wholly of ribonuclease A.

Haber (117) has applied this technique of reduction and reoxidation to the subtilisin-modified protein of Richards (111). He has found that, if the polypeptide-protein mixture is subjected to the reduction and reoxidation procedure, results similar to those of White (114) are obtained. If, on the other hand, the isolated protein part of the molecule (there is no cystine in the peptide portion) is so treated and then mixed with the peptide, no activity is regained. This suggests that perhaps the presence of the peptide protects a certain amount of vital tertiary structure so that not all the original conformation of the molecule is lost on reduction; this would result in a relative specificity in the reoxidation process. Sela & Lifson (96) have carried out a statistical analysis of the formation of disulphide bridges by oxidation of —SH groups in a randomly kinked chain. They conclude that in freshly oxidized ribonuclease some tertiary structure remains or, alternatively, other constraints operate which heavily favor specific recombinations.

There had previously been indications of a gradual conformational change in ribonuclease after reduction. White & Anfinsen (115) noted that, although there was no difference in the extent of reduction of ribonuclease left in the urea-thioglycollate reducing medium for either 4.5 or 24 hr., recovery of the active enzyme was considerably less in the latter case. In addition, Resnick, Carter & Kalnitsky (118) reduced ribonuclease at different urea concentrations. With 6 *M* urea 3.9 of the 4 disulphide bonds were reduced, yet the protein retained some 30 per cent of its enzymatic activity, whereas in 8 *M* urea both reduction and loss of activity were complete. The effect of the increased

urea concentration was greater than could be accounted for by the relatively small increase in the number of $-S-S-$ bonds reduced and presumably arose from changes in the tertiary structure. Connected with this is the observation some time ago by Harrap & Woods (119) that the viscosity of reduced wool keratins in aqueous solution changed with time in a manner suggestive of a slow change in the tertiary structure of the molecule after reduction.

Brown and co-workers (120, 121, 122) have found that the antigenic activity of ribonuclease is more sensitive to disulphide bond breakage than the enzyme activity. Although very little loss in enzyme activity accompanies the fission of only one of the four $-S-S-$ bonds, there is considerable loss of antigenicity. When only three disulphide cross-links are destroyed, the antigenicity is completely eliminated, although the molecule retains a good deal of enzyme activity.

Ginsburg & Schachman (123) have investigated the digestion of ribonuclease by pepsin in terms of enzyme activity, molecular weight (by sedimentation), and reducibility of disulphide bonds. During the initial stages of digestion, the molecule became more asymmetric, but there was little change in molecular weight and no loss in activity. Addition of mercaptoethanol at this stage resulted in the release of a small peptide, indicating that the initial attack by the pepsin was on the cyclic part of the ribonuclease molecule, which is maintained by disulphide bridges. As digestion proceeded, the distribution of molecular weights in the digest became larger and the cystine cross-links gradually became more susceptible to reduction by mercaptoethanol in the absence of urea. This behavior is consistent with breakdown of the molecule by a "zipper" mechanism and may be contrasted with the digestion of insulin by chymotrypsin (124), wherein one molecule at a time is completely digested—no molecules intermediate in size between insulin and the final peptides can be detected in a partial digest. In the pepsin-ribonuclease system, Ginsburg & Schachman (123) found that the partial digest retained a measure of enzyme activity which apparently resided in particles of molecular weight *ca.* 10,000. It seems therefore that a substantial part of the ribonuclease molecule is not essential for enzyme activity.

Physical chemical aspects of the secondary and tertiary structure.—Further data on the deuterium-hydrogen exchange properties of ribonuclease have been presented by Schildkraut & Scheraga (125). Of the 245 exchangeable hydrogens in the molecule, 175 exchanged with deuterium almost instantaneously at 0°C. Of the remaining 70, only 50 exchanged at 38°, even after long periods, and the final 20 exchanged only at temperatures of 60° or above, that is to say, under conditions in which the protein is thought to be completely unfolded (126, 127). This last result is in apparent disagreement with earlier work by Hvidt (128), who observed complete replacement of all exchangeable hydrogens at 38°. This discrepancy may be explicable from data presented by Stracher (129), who found that an old sample of ribonuclease which had been stored in the cold room over a number of years had 225 exchangeable hydrogens at 38°, whereas a new sample exchanged 245 hydro-

gens at the same temperature. Stracher attributes the retention of the 20 extra hydrogens in the old sample to an inaccessibility brought about by aggregation of the protein. A further complicating factor may be the heterogeneity of the protein: Schildkraut & Scheraga (125) have shown that the ribonuclease fraction crystallizing in the so-called form III (130) exchanges hydrogens at an appreciably slower rate than the more usually investigated form II.

Interest has been shown in the conformational changes which ribonuclease undergoes in different solvent media. Subsequent to the observation (131) that the protein takes on a more helical configuration in 2-chloroethanol, Weber & Tanford (132) investigated the changes in optical rotation and viscosity as the composition of the solvent system 2-chloroethanol-water was varied. On addition of small amounts of 2-chloroethanol (*ca.* 10 mole per cent) to the aqueous solution, there was comparatively little change in the optical rotatory properties but a large increase in intrinsic viscosity, the latter being very dependent on the ionic strength of the solution. This was interpreted as indicating a transition from the native protein, which (as judged by its optical rotatory dispersion) has a low helical content with tightly folded non-helical regions, to a form with much the same helical content but unfolded non-helical regions. This random form is thought to appear because of the suppression of non-polar side-chain interactions by the 2-chloroethanol. On addition of more 2-chloroethanol the optical rotatory properties changed in the direction of increasing helical content. At the same time, the intrinsic viscosity decreased slightly and lost its dependence on ionic strength, a characteristic of a rigid molecule. In pure 2-chloroethanol, which promotes the formation of intramolecular peptide hydrogen bonds, the protein assumed maximum helical configuration. These changes were shown to be reversible and not related to any aggregation of the protein.

Kalnitsky *et al.* (133) have studied the influence of conformational changes on the enzyme activity of ribonuclease. They have shown that when the composition of the water-2-chloroethanol system corresponded to maximum unfolding [i.e. the maximum in the viscosity-composition curve of Weber & Tanford (132)] the activity of the enzyme was reduced to about 50 per cent of its value in aqueous solution. Kalnitsky *et al.* pointed out that this loss in activity might be caused by either a structural change in the protein or a specific solvent effect of the 2-chloroethanol. They measured the rate constants, k , for the enzyme-substrate reaction in aqueous solution at 10° temperature intervals from 30°–60°C. and found that the ratio $k_{40°}/k_{30°}$ was almost identical with $k_{60°}/k_{40°}$, so that there was no anomalous temperature effect as such. On the other hand, the ratio $k_{60°}/k_{30°}$ was much lower, and it is in this temperature range that the molecule unfolds, as indicated by an increase in both the intrinsic viscosity and the laevorotation. Thus, the reduction in enzyme activity is attributed to a change in secondary structure, and Kalnitsky *et al.* argue that this, rather than a specific solvent effect, brings about the loss of activity in the 2-chloroethanol-water system. However, the optical rotation measurements of Weber & Tanford indicate that addition of

2-chloroethanol is attended by a slight increase in helical content, so it would seem that it is the perturbation of the tertiary structure which leads to inactivation. Kalnitsky & Resnick (134) have shown further that the "heat of inactivation" of the enzyme is substantially less than the heat of denaturation. It is reasonable to conclude that less change is required in the conformation of the molecule to inactivate it as an enzyme than to break down its secondary structure. This suggests again that inactivation in the mixed solvent system is not related to any changes in secondary structure.

The thermal transition at about 60° has been studied in D₂O and H₂O by Hermans & Scheraga (13), who used optical rotation to follow the conformational changes. They found that the transition temperature (i.e., that at which the transition is half complete) was about 4°C higher in D₂O than in H₂O [cf. also Calvin, Hermans & Scheraga (137)].

Foss & Schellman (135) have investigated the influence of urea on the thermal transition of ribonuclease, again using the technique of optical rotation. They showed that the addition of urea resulted in an increase in the laevorotation at room temperature and, hence, a decrease in helical content. As might be expected, the thermal transition temperature was also lowered. An unusual aspect of the results lay in the temperature-dependence of $[\alpha]_D$ at temperatures below the transition region. In the absence of urea, $[\alpha]_D$ was almost independent of temperature in this range, but when urea was present it became more positive on heating. In the simplest terms, this indicates that the protein molecule first becomes more helical on heating before eventually undergoing a transition to the fully unfolded form. This inverted transition is similar to that observed by Doty & Yang (136) when they heated poly- γ -benzyl-L-glutamate in an ethylene dichloride-dichloroacetic acid mixture. The explanation is the same in both cases: in urea solutions the random regions of the protein are stabilized by binding urea molecules (with a consequent large decrease in translational entropy) at sites required for hydrogen-bonding in the helix. As the temperature is raised the ensuing decrease in urea-binding favors helix formation. At still higher temperatures, the usual helix-coil transition takes place. The thermal coefficient of these transitions is determined by the balance between the entropy and heat changes involved in the interactions of the two solvent components and the protein itself.

The hydrogen-ion titration curves for ribonuclease in a solvent which favors the random-coil conformation have been determined by Cha & Scheraga (151). The solvent was an aqueous solution, 1.2 *M* in urea and 5 *M* in guanidine hydrochloride. It was chosen because the pK values of the ionizing groups on the protein differ only very slightly in this solvent from those in 0.15 *M* KCl, so that a direct estimate of the influence of the coil-promoting solvent on the titration curve is possible. In dilute salt solution the pK values for three of the six phenolic groups on the tyrosine side chains were abnormally high and those for the carboxyl groups abnormally low (*v.i.*). In the mixed guanidine-urea solvent, however, all the phenolic and carboxyl groups became normal. Cha & Scheraga attributed the abnormal pK values of the phenolic and carboxyl groups in 0.15 *M* KCl to internal hydrogen bonding

between these groups, which is eliminated in denaturing solvents. They also point out that the conformation of the molecule in the mixed solvent is not dependent upon pH since the electrostatic interaction factor w is constant over the whole pH range.

The involvement of tyrosyl groups in interactions relevant to the internal structure of proteins has been deduced from spectrophotometric data, and it has been widely suggested that the presence of tyrosyl-carboxylate hydrogen bonds could explain many of the observed results. A good deal of information has recently accrued on this subject, as on the nature of ultraviolet spectral changes in proteins generally. It seems appropriate, therefore, to survey this material in relation to proteins in general.

ULTRAVIOLET SPECTROSCOPIC SHIFTS IN PROTEINS

It has long been known that the spectra of proteins in the region 240 to 320 $m\mu$ differ in detail from those of the constituent aromatic amino acids (138, 139). Consequently, the hydrolysis of a protein, whether complete or partial, is attended by small spectroscopic shifts. Somewhat similar effects are also noted on denaturation, and, of late, these have been intensively studied for a number of proteins in the hope of associating them with the alteration of specific features of the secondary or tertiary structure. In order to achieve greater sensitivity in recording small changes, the technique of difference spectroscopy has been used (140); for instance, the spectrum of the denatured protein at high concentration is measured against the native protein under standard conditions at the same concentration. Differences which might be vanishingly small at concentrations appropriate for conventional spectrophotometry are then magnified. The importance of guarding against instrumental artifacts arising from stray light when the absorbance in both sample and reference is very high cannot be overstressed. These are very well-illustrated by Fridovich *et al.* (141). In addition, the problem of macromolecular scattering, which may arise when differences in state of aggregation exist between sample and reference, has been treated by Leach & Scheraga (142), who use a logarithmic extrapolation to effect a correction.

When a protein is made alkaline, a new feature appears around 294 $m\mu$ which is attributable to formation of a phenolate chromophore by the ionization of the tyrosine. This provides a ready means of titrating and estimating tyrosyl residues. In 1943, Crammer & Neuberger (143) performed spectroscopic titrations of insulin and ovalbumin and found that in the latter only two of the ten tyrosyl groups were ionized at pH 12. At higher pH the remaining groups suddenly ionized, and, since in ovalbumin which was exposed to pH 13, neutralized, and then titrated all the groups behaved normally, it could be concluded that ionization of the eight anomalous residues was made possible only by denaturation of the protein. This behavior was ascribed to hydrogen-bonding of the phenolic hydroxyls, probably to carboxylate groups.

Similar anomalous tyrosyl residues have since been reported for ribonuclease (144, 145) (in which three out of six titrate normally), for tobacco

mosaic virus protein (146), for taka-amylase A (147), and for myosin and L-meromyosin in which 35 and 90 per cent, respectively, of the residues are abnormal (in H-meromyosin all tyrosyls titrate normally with a pK_a of 10.4) (148). It was found that all tyrosyl groups in guanidine-denatured ovalbumin (149) and in ribonuclease in 8 *M* urea (150) titrated normally. Moreover, titration data at low pH for ovalbumin (149) and ribonuclease (151) indicated the presence of anomalous carboxyl groups equal in number to the anomalous tyrosyls and also normalized in the denaturing solvent. This was regarded as strong support in favor of the tyrosyl-carboxylate hydrogen-bonding hypothesis, and further evidence, based on the spectroscopic shifts which accompany denaturation, has been adduced for a number of proteins.

One instance has very recently come to light of anomalous tyrosine residues, which ionize only above pH 11.5 but with reversible disruption of the protein structure. Smillie & Kay (152) have found that four of the nine to ten tyrosyl residues in trypsinogen are normal and four more are reversibly exposed at a high pH, with a concomitant change in the tertiary structure, if the protein is maintained at 10°. At 25 and 37°, however, the change is irreversible and may involve the liberation of the remaining groups.

In the difference spectra of proteins such as ribonuclease (153, 154) and insulin (155), which contain no tryptophan, the now well-known "denaturation blue-shift" manifests itself in the form of two peaks at about 279 and 287 μ . Laskowski *et al.* (155) initially applied difference spectroscopy to follow the tryptic proteolysis of insulin by measuring the spectrum of a tryptic digest against a reference solution of the native protein at the same concentration. The characteristic two-banded difference curve for tyrosine was obtained [cf. the similar result reported (156) for the peptic digestion of ribonuclease] and was evidently attributable to a change in the environment of the tyrosyl residues on proteolysis. This is consistent with the liberation of a tyrosine-containing peptide on tryptic hydrolysis of insulin. The change was ascribed more specifically to the rupture of a tyrosyl hydrogen bond as the peptide was liberated. Laskowski *et al.* (155) also determined the difference spectrum of a neutral insulin solution against one at pH 2 where the carboxyl groups would be protonated, and they again observed the characteristic two-peaked spectrum. By measuring the absorbance difference at 287 μ as a function of pH, a "titration" curve was obtained which was attributed to the ionization of the acceptor group of the tyrosyl hydrogen bond. The apparent pK was about 3, which might correspond to a hydrogen-bonded carboxylate group. Laskowski, Leach & Scheraga (157) subsequently found that the shifts due to acidification and tryptic digestion occurred independently of one another and that two tyrosyl groups were thus involved; one, it was suggested, was hydrogen-bonded to an ionizing donor (carboxylate), the other to a non-ionizing donor.

Scheraga (153) found, similarly, a pK of about 2 for the putative acceptor group in ribonuclease. This value, however, is very low, and the slope of the titration curve is anomalously steep. To account for these observations, Scheraga proposed a mechanism whereby the acceptor group is involved in a

hydrogen bond of the acetic acid dimer-type when in the unionized state at low pH. At higher pH it becomes an acceptor in a co-operative hydrogen bond with two tyrosyl groups.

Glazer, McKenzie & Wake (158) observed a similar change in the tyrosyl spectrum in bovine serum albumin; the change accompanied the low-pH transition of the protein into the "expanded" form and also occurred in the presence of urea. Williams & Foster (159) investigated the difference spectrum of bovine serum albumin as a function of pH, ionic strength, anionic species present, and urea concentration. They found that the changes in the magnitude of the difference maxima did not parallel the protonation of carboxyl groups; indeed, circumstances favoring protonation at a given pH brought about a decrease rather than an increase in the difference peak. It follows then that either the hydrogen-bond acceptors for the tyrosyl residues are not carboxylate groups or that tyrosyl-carboxylate hydrogen bonds are broken as a consequence of molecular expansion rather than by pH changes as such. Alternatively, the difference spectra might arise from a change in polarizability—and thus refractive index—of the environment of the chromophore when the protein expands. This concept of the mechanism has gained ground with fresh experimental results at the expense of the tyrosyl-carboxylate hydrogen-bond hypothesis.

All the proteins so far discussed contain little or no tryptophan. Examination of lysozyme (160), pepsin (161, 162), chymotrypsin, and chymotrypsinogen (163) revealed the onset on denaturation of a band, attributable to tryptophan, at about 292 to 294 $m\mu$ in the difference spectrum. In chymotrypsin, for instance, this was found to appear during autolysis and urea denaturation, whereas the chymotrypsinogen-chymotrypsin conversion was attended by a change associated almost exclusively with the tyrosyl bands. It will be noted that any change involving the tryptophan residues must necessarily arise from an environmental effect since the chromophore itself can undergo no modification.

Wetlaufer, Edsall & Hollingworth (164) have demonstrated with the aid of model compounds that the shifts in tyrosyl absorption which give rise to the characteristic difference spectra may be reproduced merely by the ionization of vicinal groups and also by the addition of urea or sodium acetate. It was suggested that the latter reagents operate by dipole-dipole interactions with the chromophores, but, as Yanari & Bovey (165) have pointed out, such interactions would result in greater solvation energies in the ground states than the excited states and, consequently, a blue rather than a red shift in the spectrum.

Bigelow & Geschwind (166) showed that the spectra of proteins, as well as model compounds, underwent marked red shifts in media of high refractive index, such as strong salt and sucrose solutions. Urea at concentrations too low to bring about denaturation produced a similar shift, whereas at higher concentrations the larger denaturation blue shift became superimposed on this effect. These observations are entirely consistent with the above-mentioned hypothesis that the aromatic residues concerned in the de-

naturation shifts are located in a region of very high refractive index (protein matrix) and become exposed to the solvent when the protein structure is disrupted.

Bigelow (167) has reinvestigated the spectral properties of ribonuclease in the light of these data. In order to separate the contributions of the anomalous tyrosyl residues, he has compared the native protein with per-formic acid-oxidized and pepsin-inactivated ribonuclease (168). Whereas in the native protein three of the six tyrosyls titrated anomalously, one only was abnormal in the pepsin-inactivated material, and in the oxidized form all were normal (169). Acidification gave no spectral shift for the oxidized ribonuclease, whereas both the native and pepsin-inactivated substances showed blue shifts with difference peaks of closely similar magnitudes. Only one of the anomalous tyrosyl residues in ribonuclease is involved in the acid-induced shift, and, moreover, since the other two residues are not normalized under the same conditions, they are not involved in tyrosyl-carboxylate hydrogen bonds. Indeed, on the basis of Scheraga's model in which each carboxylate ion must form a co-operative hydrogen bond with two tyrosyls, none of the tyrosyl residues in native ribonuclease can be so involved.

Bigelow has also studied quantitatively the difference spectra of ribonuclease in urea and lithium bromide. He used the data of Bigelow & Geschwind (166) for the magnitude of the red shift in non-denaturing concentrations of these reagents to correct the observed change in extinction coefficient at 287 $m\mu$ and thus to determine the net blue shift attributable to denaturation. The value obtained for urea-denatured ribonuclease was equal to that for the total peptic digest (both solutions were measured against native protein) but greater than that in 5 *M* LiBr. In lithium bromide, denaturation evidently takes place in two stages, the first at a concentration of 3 to 4 *M*, the second at 9 to 10 *M*. A plateau separates the steep increases in absorption difference occurring in these regions.

It was possible then to classify the anomalous tyrosyl residues into three types: (a) anomalous in native and pepsin-inactivated ribonuclease but not in oxidized ribonuclease and normalized by acid, 5 *M* LiBr, 8 *M* urea, and pepsin digestion; (b) anomalous only in native ribonuclease and normalized by partial peptic digestion, 5 *M* LiBr, and 8 *M* urea but not by acid; (c) anomalous only in native ribonuclease and normalized by partial digestion, 10 *M* LiBr, and 8 *M* urea.

Classifications of abnormal tyrosyl residues in ribonuclease and other proteins in terms of lability to heat denaturation have also been made by Tramer & Shugar (170).

A definitive investigation of difference spectra brought about under a variety of circumstances in proteins and model compounds has recently been carried out by Yanari & Bovey (165). The inductive effect of vicinal charges ruled out as a material factor in defining the shifts in proteins; hence, pH-induced difference spectra must be essentially attributable to changes in secondary or tertiary structure. The blue shift, associated with all the aromatic residues, which accompanies denaturation can be reproduced in

the spectra of benzene, indole, and phenol when the refractive index of the solvent is lowered, in particular when the chromophores are transferred from a hydrocarbon to an aqueous environment. The concept of protein denaturation which this picture elicits is remarkably consistent with current ideas on hydrophobic bonding in proteins (171). It would seem that the weight of evidence favors an explanation for ultraviolet spectral shifts in proteins in such terms. The affected residues are envisaged to lie in a hydrophobic cage comparable with a detergent micelle; indeed, Yanari & Bovey have found that the spectral differences between proteins in the native and denatured states can be reproduced in simple aromatic chromophores in the presence of detergent in concentrations sufficient for micelle formation.

Leach & Scheraga (172) summarize the environmental effects which might be capable of giving rise to difference spectra in proteins. They make the point that generalizations are not permissible at this stage and that data for every protein must be considered individually. They admit that the pH difference spectra for reversible denaturation can be explained in terms of hydrophobic bonding without invoking the concept of tyrosyl-carboxylate hydrogen bonds.

It has been pointed out that, if some chromophores in the protein are "exposed" while others are "buried" in a hydrophobic matrix and are inaccessible, then only the former should be subject to spectral shifts when the refractive index of the medium is changed in the absence of conformational changes in the protein. Herskovits & Laskowski (173) have observed the difference spectra brought about by red shifts in the presence of hydroxylic solvents (20 per cent sucrose, glycols, etc.). In native ribonuclease and bovine serum albumin only 50 per cent and 30 per cent, respectively, of the tyrosyl residues were found to be affected if comparisons were made with the protein in the random-coil state (generated by reduction of all disulphide bridges), in which the difference peak is increased by the contributions of the remaining tyrosyls.

It would seem that this type of investigation may lead to useful data on internal protein interactions. It may be compared with experiments such as those of Williams & Foster (174) and Klotz and associates (175, 176) on the behavior of extraneous groups introduced into the protein environment. Anthracene coupled to bovine serum albumin (174) leads to a pH-dependent difference spectrum the behavior of which is strikingly reminiscent of the tyrosine difference spectrum for the same protein. The shift is evidently related to the expansion of the protein molecule at acid pH, and it seems likely that a change in local polarizability may be responsible for the observed effects. Again, when a basic conjugate is similarly coupled to the albumin (176), a shift in pK of no less than 2.3 units is observed; this shift is almost nullified when the protein is denatured with urea.

Finally, the recent findings of Glazer & Smith (177) must be mentioned: difference spectra for a variety of proteins, variously denatured, against a reference native protein showed, in addition to the previously described features, a much larger band at 230 m μ which was unrelated in magnitude to

the content of chromophoric amino acids. This may almost certainly be equated with the intersections at about 218 and 243 $m\mu$ of the absorption curves for the helical and random forms of polypeptides. The essential difference between the spectra of these configurations is a substantial hypochromic effect in the helix which was first noted for poly-L-glutamic acid by Imahori & Tanaka (178). Although, in proteins generally, the changes in the short-wave ultraviolet spectrum, which are associated with denaturation, cannot be identified with a simple helix-coil transition, the changes are certainly dependent on alterations of some kind in secondary structure. Data for polypeptides and proteins have been compiled and are fully discussed by Rosenheck & Doty (179).

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SPECIFICITY IN PROTEIN SYNTHESIS^{1,2,3}

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The biological activity and structural uniqueness of proteins depend on the three-dimensional configuration assumed by the polypeptide chain. Each molecular configuration is, in turn, a function of the sequence of amino acids along the chain and of the way in which these amino acids interact with one another. The mechanism of protein synthesis must therefore provide for the formation of specific amino acid sequences and the correspondingly specific three-dimensional configuration of the polypeptide backbone. In the present paper, I will consider some of the data and hypotheses which relate to the mechanism by which this specificity is assured. For additional discussions of protein biosynthesis the reader is referred to several recent reviews (1 to 17), reports of symposia (18, 19, 20), and individual papers (21, 22).

THE CONTROL OF AMINO ACID SEQUENCE AND PROTEIN STRUCTURE BY DNA

The most widely accepted hypothesis for the participation of DNA in protein synthesis proposes that the information necessary for the production of specific amino acid sequences is encoded in the nucleotide sequence, a particular length of the DNA [a cistron (23, 24)] corresponding to a given polypeptide chain (see 4, 6, 7, 8, 18, 19, 20). This theory explicitly assumes that no information other than that required to specify the amino acid sequence is necessary to form the appropriate molecular configuration (25). In this section, I shall summarize the evidence which supports this view and, in addition, consider the question of whether DNA is involved directly or indirectly in the specification of amino acid sequences.

¹ Most of the literature cited in this review appeared before December 1, 1960, although several papers were in press at the time of writing and have appeared subsequent to that date.

² The following abbreviations are used: AMP for adenosine monophosphate; ATP for adenosine triphosphate; ATPase for adenosine triphosphatase; CTP for cytidine triphosphate; DNA for deoxyribonucleic acid; DNase for deoxyribonuclease; GTP for guanosine triphosphate; PP for inorganic pyrophosphate; RNA for ribonucleic acid; RNase for ribonuclease; TMV for tobacco mosaic virus.

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THE CONTROL OF AMINO ACID SEQUENCES BY DNA

Since several excellent reviews, with extensive bibliographies, of the mechanism of gene action have appeared recently (4, 6, 7, 8, 18, 19, 20, 26), I will forego the citation and detailed discussion of individual papers dealing with this subject. I will, however, summarize the major conclusions as they relate to the question of specificity.

The implication of DNA as the genetic carrier of the information for specification of protein structure originates from a variety of studies. Alteration of the cellular genotype by mutation, bacteriophage infection, conjugation and genetic recombination, or transformation with free DNA leads to a phenotypic modification in the affected cell. In most cases this modification results from a loss or gain in the potential to form a specific protein [see (4, 6, 7, 8) for references to specific papers]. These and other studies focused attention on the primary role of DNA in the control of protein synthesis. Genetic fine structure analysis of a single functional gene, gave credence to the concept that the information carried by the gene was distributed linearly along the genome in a fashion which presumably corresponds to the nucleotide sequence in DNA.

The evidence that alterations in DNA structure result in modifications of amino acid sequences comes mainly from studies of the effect of mutations on the structure and biological activity of enzymes. Deletion or point mutations within a particular genetic element usually result in a structural modification of the corresponding protein and very often in an alteration of its specific biological property (see 6, 8, 18, 20, 26). Such structural changes in proteins from mutants have been detected as changes in net charge of the protein, as alterations in the sensitivity to inactivation by a variety of conditions, and by immunochemical cross reactions (see 6, 8). The chemical basis for such structural changes was first established in the analysis of the human hemoglobins. In each of the cases analyzed, a single mutation led to the replacement of one amino acid by one of several others. The successes with the hemoglobins have stimulated similar studies with systems more amenable to extensive genetic manipulations and from which a large variety of mutant proteins may be obtained. In particular, microbial systems currently under intensive study are tryptophan synthetase, alkaline phosphatase, β -galactosidase, T4 phage head proteins, and the T4 phage-induced lysozyme. In each case altered proteins have been isolated from mutant organisms; the proteins so derived range from nearly zero to almost normal in functional activity. At present only the mutant tryptophan synthetases [(6) and related papers quoted therein], T4 phage head proteins (27), and the T4 phage-induced lysozymes (28) have yielded differences in the peptide patterns obtained after partial enzymic digestion.

These and other studies are progressing toward the extensive mapping and identification of the defects in the amino acid sequences of mutant proteins. There may then be an answer to the question of whether the linear order of the mutations determined by genetic mapping corresponds to the

order of amino acid changes in the polypeptide chain. However, while these studies provide an encouraging start to an understanding of the coding mechanism, i.e., the correspondence between amino acid and nucleotide sequence, it is only part of the problem. Almost completely unexplored are methods for detecting changes in the nucleotide sequences of DNA. The only progress along these lines has been a statistical analysis of the frequency of neighboring nucleotides (29, 30, 31). It has been suggested (6) that the mutagenic action of certain purine and pyrimidine analogues (32 to 35) might serve to characterize certain types of mutations and thereby bypass part of the need for detailed nucleotide analyses. Possibly the use of chemical reagents, such as nitrous acid (36 to 39) and alkylating agents (40), to modify nucleotide structures within the DNA may prove to be an alternative to the more laborious determinations of nucleotide sequence. However, the lack of specificity of such reagents argues against this approach.

IS THE AMINO ACID SEQUENCE SUFFICIENT TO SPECIFY THE CONFIGURATION OF A PROTEIN?

The "sequence hypothesis" predicts that a polypeptide chain that has its amino acids arranged in a specific order will assume a unique three-dimensional configuration (25). The related question of whether a native protein possesses a unique molecular configuration is presently unanswerable (41). By use of the reversible boundary spreading technique (42, 43) as an indicator of molecular heterogeneity, none of the 14 purified proteins tested, including seven crystalline preparations, could be characterized as homogeneous (44). Whether this is attributable to subtle configurational and charge differences amongst molecules which have an identical amino acid sequence is not yet clear (41, 44). Nevertheless, we may consider the "sequence hypothesis" in more operational terms and rephrase the original question to ask whether the amino acid sequence dictates the formation of a three-dimensional structure with a specific biologic activity.

After complete inactivation of RNase activity by reduction of the four disulfide linkages, almost 80 per cent of the original specific activity is restored by reoxidation (45). Physical and immunologic studies of the reoxidized product indicate that the recovered material closely resembles the original preparation (46). Since 105 isomers can result from the random reoxidation of the sulphydryl groups (47) [the structure existing in RNase is neither the most nor the least probable (47)], the observed result implies a high degree of oriented specificity in the reformation of the specific configuration.

Studies with tobacco mosaic virus (TMV) protein have shown that, following denaturation by phenol, treatment with strong alkali, or exposure to 8 *M* urea, protein can be recovered which in the presence of TMV-RNA aggregates to yield normal infectious virus particles (48). In this particular case no disulfide bridges need be reformed.

Additional examples of oriented reformation of native structures from

denatured protein come from experiments with human serum albumin (49) and insulin (50). Following reduction of the 17 disulfide linkages, the specific combining capacity of human serum albumin with antibody was inactivated. After reoxidation, approximately 50 per cent of the combining activity was recovered (49). Similarly, after reduction of insulin and separation of the reduced α - and β -chains no biological activity was demonstrable with each fraction alone, either before or after reoxidation of the individual components. Mixing the α - and β -chain preparations, under conditions whereby disulfides are regenerated, resulted in the reformation of native insulin in approximately 1 to 2 per cent yield (50).

These data therefore appear to be consistent with the "sequence hypothesis." Those instances wherein recovery of native protein following reductive denaturation and reoxidation is either not observed or is low (50, 51, 52) may be a reflection of the particular conditions employed. For example, the reformation of native insulin from the two reduced chains would be expected to be concentration-dependent. In this connection, one need only consider the recent success in reforming native double-stranded DNA from single strands produced by heat denaturation (53) as an example of how subtle the correct conditions for renaturation may be. Nevertheless, the question of whether the biological mechanism of protein synthesis relies on spontaneous folding to produce specific protein structures remains unanswered. It is possible that in the process of polypeptide chain synthesis constraints exist which favor certain modes of formation and not others.

IS DNA DIRECTLY INVOLVED IN SEQUENCE SPECIFICATION AND POLYMERIZATION OF AMINO ACIDS?

I refer to the question of whether DNA acts directly as a template or indirectly through the formation of an intermediate template; in each case I use the term template to refer to a structure which directs the formation of the precise sequence of amino acids for a given polypeptide chain (54, 55).

The view that DNA acts indirectly in protein synthesis developed from several types of experiments. For example, enucleated cell fragments of *Amoeba proteus* and *Acetabularia mediterranea* continue to incorporate labeled precursors into the cytoplasmic proteins (56 to 58). With *Acetabularia* measurable increases in protein nitrogen continue after removal of the nucleus (59), and certain enzyme activities also increase (60). Another example is the synthesis of hemoglobin by non-nucleated reticulocytes (61, 62). The experiments cited above, however, deal with the question of whether an intact nucleus is essential for cytoplasmic protein synthesis. If there is any extranuclear DNA, these experiments do not eliminate the possibility that DNA may function directly as the template. It is crucial to establish a lower limit for the level of DNA in the cytoplasm of such non-nucleate fragments or cells. For example, reticulocytes do contain DNA (63), and tritiated thymidine is incorporated by *A. proteus* into an acid-insoluble material

within the cytoplasm that is DNase-sensitive and RNase-insensitive (64). While this is by no means proof that this cytoplasmic material is functional DNA, it raises the issue of whether a cytoplasmic DNA component is involved in protein synthesis.

So far, studies with cell-free systems have not answered this question either. The original observation that DNA stimulated amino acid incorporation and enzyme formation by disrupted *Staphylococcus aureus* preparations (65, 66) loses its significance because of later findings that unidentified non-nucleotide "incorporation factors," present in the nucleic acid preparations, are responsible (66).⁴ Similarly, the effect of DNA in stimulating amino acid incorporation into proteins of isolated thymus nuclei (67) is now known to be attributable to indirect effects of DNA (or any nucleic acid) in promoting ATP-formation by oxidative phosphorylation (68). The effect of removing DNA on β -galactosidase formation by osmotic lysates of *Bacillus megaterium* spheroblasts (69) is irrelevant because DNA was resynthesized under the conditions used to study enzyme formation (69).

Studies of amino acid incorporation into proteins by means of partially purified systems yield no clear evidence of a requirement for DNA. Few, if any, analyses for DNA in the soluble, microsomal, or submicrosomal fractions have been made. In most studies, the effect of RNase is routinely examined, but tests for the action of DNase on the rate and extent of incorporation are rare (e.g., 70 to 73). Recently, however, relatively high levels of pancreatic DNase were found to result in a marked inhibition of amino acid incorporation (74) and of β -galactosidase synthesis (75) by cell-free systems from *Escherichia coli*, but the critical demonstration of the restoration of the activity by the addition of DNA has not been supplied.

Additional experiments related to the role of DNA in protein synthesis have come from studies of the kinetics of gene expression in zygotes formed by conjugation of bacterial cells. Following introduction of the σ^+ gene (the genetic region which controls the structure of β -galactosidase) into a σ^- recipient cell, there is no apparent delay in attainment of the maximal rate of enzyme synthesis by the zygote (76, 77). Somewhat similar measurements of the kinetics of formation of specific enzymes following infection with T-even phages indicate that less than 4 min. is required for detectable amounts of the enzymes to be formed (78 to 83). These experiments indicate that, if DNA is not the template, the time necessary for template synthesis and expression is relatively short. Nevertheless, the results described above do not permit a clear choice between the direct and indirect hypotheses of DNA action.

If intermediate templates are made in response to the DNA, the evidence suggests that they are not stable; i.e., they do not persist within the cell. Thus, although entrance of the σ^+ gene into the recipient σ^- cell [in a

⁴ These "incorporation factors" appear to be cytosine- and uracil-containing derivatives of glycerol (263).

ming of $P^{32}-z^+$ (Hfr) \times $P^{31}-z^-$ (F^-)] results in almost immediate formation of enzyme, progressive P^{32} decay of the z^+ genome in the early zygotes leads to loss in the ability to produce β -galactosidase (77). These experiments are in agreement with earlier conclusions that the continued integrity of the DNA is essential for the expression of its phenotype (84). Moreover, they support the view that intermediate enzyme-forming templates, if they exist, disappear in the absence of the controlling gene. It should be pointed out that, since few, if any, of the z^+ loci sustain a P^{32} decay (84, 85), the integrity of the entire injected genome appears to be essential for the expression of the z^+ locus.

The simple interpretation that entry of a new gene into the recipient cytoplasm immediately initiates its expression is questionable. For example, if in an experiment similar to the one described above the P^{32} label of the cells is reversed [i.e., $P^{31}-z^+$ (Hfr) \times $P^{32}-z^-$ (F^-)], decay of the injected z^+ enzyme-forming potential also occurs, and the extent to which this potential is lost is a function of the amount of P^{32} in the z^- recipient (86). Thus, it would appear that not only must the incoming gene be conserved for its expression but also the recipient genome must remain intact. While these findings can be interpreted as reflecting the necessity of some form of interaction between a newly introduced gene and the recipient genome, further work is necessary to clarify this point.

I would summarize the preceding discussion with the following statements: (a) DNA very likely acts in protein synthesis by specifying amino acid sequences and this may be sufficient also to determine the configuration of the protein; (b) while nuclear DNA may be eliminated, the evidence concerning a direct role of DNA as the template is indecisive. The experiments with bacteria indicate a requirement for the continued integrity of the DNA (insofar as P^{32} decay alters the integrity of the DNA) for protein synthesis. In spite of increasing indications that RNA functions as an intermediate template for specifying amino acid sequence (see section on function of RNA), the exclusion of a direct role for DNA is premature.

CONTRIBUTIONS OF RNA TO THE FORMATION OF SPECIFIC AMINO ACID SEQUENCES IN PROTEINS

Although DNA has not been rigorously excluded from direct participation in polypeptide synthesis (see previous section), there is considerable evidence that RNA is an essential component in the process. Much of the literature which establishes the participation of RNA has already been reviewed (1, 11, 12, 15, 16); therefore, few of the early papers will be discussed here. The first clear indication that RNA, like DNA, can carry information for specifying protein structure was obtained with isolated RNA from TMV (87, 88). It was observed, and subsequently confirmed, with RNA from a variety of animal and plant viruses [see (4) for review of these papers] that infection of suitable hosts with RNA alone provoked the syn-

thesis of new and complete virus nucleoprotein particles. Deamination of a portion of the TMV-RNA nucleotides with nitrous acid resulted in the production of mutant virus particles (89); in one instance the mutation was accompanied by an alteration in the amino acid sequence of the virus protein (90). On this basis and by analogy with the mechanism proposed for DNA participation in protein synthesis, a coding scheme which involves a functional correspondence between ribonucleotide and amino acid sequences is also assumed to exist.

In this section I propose to examine in detail more recent investigations, notably with partially purified cell-free preparations, which have led to a clearly defined hypothesis for the function of RNA in the intermediate reactions of protein synthesis. The outline of this hypothesis (1, 25) is as follows: Each amino acid is activated by conversion to a specific amino acyl RNA derivative. The amino acyl RNA compound, either as such or after modification, is enzymatically transferred to cytoplasmic ribonucleoprotein particles where the amino acids are arranged with respect to their eventual sequence in the protein to be made. This process of sequence specification occurs by base-pairing through hydrogen-bonding between the amino acyl RNA and a complementary segment of RNA contained within the nucleoprotein particle. Peptide-bond formation between these specifically oriented amino acids occurs at the expense of the free energy change which accompanies the cleavage of the amino acyl RNA linkage. Folding of the polypeptide chain occurs spontaneously or by an as yet unspecified mechanism. The amino acid-acceptor polynucleotides (or their derivatives), now freed of amino acids, are reutilized for additional cycles of amino acid transfer and polypeptide chain formation.

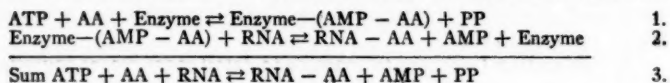
Several features of this hypothesis bear directly on the problem of the specificity in protein synthesis. These involve the mechanism for: (a) the combination of each amino acid with its appropriate acceptor RNA; (b) the recognition and interaction between amino acyl RNA derivatives and the ribonucleoprotein particle sites specific for each amino acid; and (c) the transfer of information for specifying amino acid sequences from the DNA-nucleotide order to the ribonucleoprotein particle. In the following discussion I shall consider each of these problems in turn.

THE FORMATION OF SPECIFIC AMINO ACYL RNA COMPOUNDS

Mechanism of the reaction.—According to the hypothesis stated above, amino acyl RNA formation represents the first stage in protein synthesis at which discrimination between amino acids occurs. It is of interest, therefore, to consider whether these reactions are sufficiently specific to be of significance in the specification of amino acid sequence. Before discussing the specificity features of the reactions, I shall summarize some of the pertinent details of amino acyl RNA formation.

Formation of amino acyl RNA derivatives (Reaction 3) (91 to 99) results

from the sum of two intermediate steps. The first involves the synthesis of amino acyl adenylate-enzyme complexes (Reaction 1) and the second the transfer of the amino acyl moieties to a unique class of RNA molecules (Reaction 2). The two steps are mediated by a single enzyme, and each enzyme appears to be specific for the formation of only one amino acyl RNA derivative. Each amino acid is linked to a separate and specific RNA chain,



through an ester bond, to an hydroxyl group of the ribose moiety of a terminal adenylic acid residue; whether it is the 2'- or 3'-hydroxyl group has not yet been established. The thermodynamic amino acyl transfer potential is of the same order as the pyrophosphoryl group potential of ATP.

The experimental evidence supporting these conclusions may be summarized as follows: The enzymes that catalyze amino acyl RNA formation [amino acyl RNA synthetases (97)] also promote an amino acid-dependent ATP-PP_{ss} exchange (91, 94, 96, 97, 99), an ATP-dependent formation of amino acid hydroxamates (100 to 104), and an amino acid- and RNA-dependent exchange of AMP with ATP (93).⁵ When catalytic quantities of enzyme are used, in the absence of acceptor RNA, there is no net accumulation of amino acyl adenylates (101, 102, 103); with larger amounts of enzyme, amino acyl adenylate can be detected in amounts approximately equivalent to the amount of enzyme (105, 106, 107). Chemically synthesized amino acyl adenylates are readily converted to ATP in the presence of PP (107, 108, 109) but yield amino acyl RNA in the presence of acceptor RNA (110). Despite a variety of enzyme fractionation procedures, separation of the enzyme activities for Reactions 1 and 2 has not been observed (94, 97, 99), yet the same enzyme activities for different amino acids are readily separated (98, 99, 111). The equilibrium constant for valyl- and threonyl-RNA formation ranges between 0.3 and 0.7, thus emphasizing the high-energy character of the amino acyl group (97, 99, 112, 113).

The proposed acyl ester linkage with the terminal nucleotide of the RNA is supported by the following facts. With a given amount of RNA, each amino acid is bound to a characteristic but different extent; the amount of any amino acid that becomes linked to the RNA is independent of the extent to which any other amino acid is bound (91; 94 to 99; 114). Both observa-

⁵ Recent studies (173) have shown that the amino acyl RNA derivative, γ -glutamyl cysteinyl RNA, is formed as an intermediate in the conversion of γ -glutamyl cysteine and glycine to glutathione. In the formation of γ -glutamyl cysteinyl RNA, ATP is converted to ADP and inorganic phosphate, and in this sense the mechanism differs from the amino acyl RNA synthetases under discussion (see Reaction 3). No amino acyl RNA intermediate is involved in the formation of γ -glutamyl cysteine (264).

tions are consistent with the view of separate and specific binding sites in the RNA for each amino acid. Removal or destruction of the hydroxy terminal nucleotide residue by the action of venom diesterase (112), by pyrophosphorolysis (115 to 118), or by periodate oxidation (112, 115) inactivates the acceptor activity for all amino acids tested; periodate oxidation of acceptor RNA containing a single amino acid results in the loss of acceptor activity for all but the bound amino acid (112). Consistent with the involvement of the 2'-3'-hydroxyl group of the terminal ribose is the identification of 2'-3'-amino acyl adenosine derivatives in RNase digests of amino acyl RNA (112, 265). Support for the proposed acyl linkage comes from the formation of amino acid hydroxamates when amino acyl RNA is exposed to neutral hydroxylamine (91, 94, 265) and from the close similarity in the rates of hydroxylaminolysis of amino acyl RNA and of synthetic 2'-3'-amino acyl adenosine derivatives (265).

The specificity for amino acids.—As already pointed out, the formation of amino acyl RNA compounds is specific; that is, each amino acid is linked only to its appropriate RNA chain. To what can this selectivity be attributed? Before we can attempt an answer to the question, two aspects of the reactions' specificity must be clarified. First, each enzyme must distinguish the correct amino acid from all others, and, second, it must select the appropriate RNA chain from a population of closely related molecules.

The first problem, that is, the specificity of each enzyme for a single amino acid, has not been systematically studied, and in those instances in which purified enzymes have been examined only the specificity of the ATP-PP exchange reaction or of amino acid-hydroxamate formation was tested (99, 101, 111, 119, 120, 121, 266, 267). Moreover, in most cases in which activity has been detected with more than one amino acid, few attempts have been made to determine if this was caused by contamination by other enzymes or whether it reflected the actual specificity of the enzyme. As will be seen below, the specificity, characteristic of the first step in the reaction, i.e., amino acyl adenylate formation, is not a true reflection of the specificity of the second reaction, i.e., the transfer to RNA.

Two enzymes isolated from *Escherichia coli* catalyze the formation of L-valyl RNA and L-isoleucyl RNA respectively (111). The first enzyme reacts only with L-valine and L-threonine (considering only the naturally occurring amino acids) when measured by ATP-PP exchange. Kinetic studies have established that both amino acids are utilized by a single enzyme; the K_m and V_{max} are 100 times less and three times greater, respectively, for L-valine than for L-threonine. Thus, although in this case two amino acids react with a single enzyme, the degree of discrimination is nevertheless quite large. Unfortunately, the relative reactivity of these two amino acids with regard to amino acyl RNA formation has not been tested. The importance of examining the over-all reaction, rather than a part of it, is illustrated by studies with the isoleucyl RNA synthetase (97, 111). This enzyme, although

it supports ATP-PP exchange with either L-isoleucine or L-valine (the V_{\max} with isoleucine being twice that with valine), catalyzes the incorporation of isoleucine but not of valine onto the acceptor RNA. Moreover, valine, which has a K_m about 100 times greater than that for isoleucine, competitively inhibits isoleucyl RNA synthesis. Thus we have an example wherein one enzyme, the valyl RNA synthetase, distinguishes completely between valine and isoleucine, whereas another enzyme, the isoleucyl RNA synthetase, does so only partially in the first step of the reaction but completely in the second step.

Additional evidence for a greater specificity in the second step of the reaction is seen in the transfer of L-tryptophan from the enzyme-tryptophanyl adenylate complex to RNA (122). Although a variety of synthetic D- and L-amino acyl adenylates are utilized for ATP formation with the tryptophanyl RNA synthetase (107, 123), only L-tryptophanyl adenylate is a substrate for amino acyl RNA formation (122). Thus, the enzymes which bind a variety of amino acyl adenylates [although to different extents (102, 107, 109)] appear to be considerably more specific in transferring the amino acids to RNA.

Amino acyl RNA synthetases can also utilize amino acid analogues. In most cases, however, only ATP-PP exchange or amino acid-hydroxamate formation has been studied. For example, 6-fluorotryptophan, azatryptophan, tryptazan, and 5-fluorotryptophan support hydroxamate formation and ATP-PP exchange with the tryptophanyl RNA synthetase (123). β -Methyltryptophan, 5-methyltryptophan, 6-methyltryptophan, and D-tryptophan, on the other hand, are inactive, although they inhibit the utilization of L-tryptophan (123). Those tryptophan analogues, which are substrates for the enzyme, are incorporated into cellular protein, presumably in place of tryptophan (124, 125). By contrast, several of the inactive analogues have been shown to inhibit protein synthesis without being incorporated (126, 127, 128). Additional amino acid analogues, the utilization of which by the appropriate amino acyl RNA synthetase is consistent with their incorporation into protein, are *p*-fluorophenylalanine (123, 127, 128, 129), ethionine (130, 131), selenomethionine (131, 132, 133), and norleucine (131, 132). The formation of peptidyl adenylates (134) and of D-alanyl adenylate (135) has also been reported. It is important, however, that the utilization of amino acid analogues, peptides, D-amino acids, etc., be confirmed and extended to studies of amino acyl RNA formation. Only in this way will it be possible to determine if the amino acyl RNA synthetases account for the selectivity in excluding unnatural amino acids from proteins [see (3, 41) for additional discussions of the incorporation of amino acid analogues into proteins in a variety of biological systems]. In this regard, alloseleucine has been shown to compete with valine and isoleucine for their respective RNA chains (136). The extent to which alloseleucine can replace valine and isoleucine on the RNA is, however, not established.

The specificity for acceptor RNA chains.—As pointed out earlier, each amino acyl RNA synthetase reacts only with a specific acceptor RNA chain. In each of the RNA preparations examined thus far, the acceptor end of each polynucleotide chain is terminated by an identical trinucleotide sequence, adenylyl-5'→3'-cytidylyl-5'→3'-cytidylyl-5'→3'-RNA (RNA ··· pCpCpA) (116, 117, 118). Moreover, the non-acceptor end of each RNA chain is a guanosine-5'-monophosphate (114, 137). It is likely, therefore, that the structural features contributing to the specificity reside in the interior of the polynucleotide chain. In attempts to define the features responsible for the specificity of the acceptor RNA, several parameters of the RNA structure have been examined.

Estimates of the average chain length of acceptor RNA isolated from mammalian (91, 98, 137, 138, 139), yeast (140, 141, 142), and bacterial (114, 143, 144, 145) sources have shown some variation. Based on the amount of adenosine obtained by alkaline hydrolysis (the adenosine is derived from the hydroxy terminal adenylic acid), most of the values are between 75 to 100 nucleotide residues per chain, consistent with a molecular weight of 25,000 to 35,000. In two instances, chain lengths of about 40 (146) and 60 (114) nucleotides have been reported. In one of these, however (146), all four naturally occurring nucleosides, rather than only adenosine (144, 155), were found after alkali hydrolysis. In the latter studies (114), physical measurements indicated an *S* of 4.6 and a weight-average molecular weight of about 35,000, or about twice that indicated by end-group analysis. Lower values for the average chain length may be attributed to the presence of non-acceptor oligo- and polyribonucleotides or degraded acceptor RNA chains.

The original published value for the sedimentation coefficient of liver acceptor RNA was 1.85 *S* (91), but more recent studies with similar material (139) and with preparations from yeast (140, 141, 142) and bacteria (114, 143, 144, 147) have given *S* values of about 3.5 to 4.5. In conjunction with diffusion and viscosity data (114, 143), the weight-average molecular weights have been estimated to be in the range of 25,000 to 30,000. Recent studies with acceptor RNA which contained bound amino acids (isolated in this form) reported a sedimentation coefficient of 2.7, although the molecular weight determined by sedimentation equilibrium was about 25,000. Removal of the bound amino acids with alkali produced an increase in the *S* to 4 but no change in molecular weight. This increase in *S* has been interpreted as the result of a change from a relatively extended shape to a compact configuration. X-ray diffraction studies appear to be consistent with the view that the native RNA is a "rodlike" double helix. The significance of the collapse in molecular structure after removal of the amino acids is not clear; studies of the effect on *S* of adding back amino acids *in vitro* were not made. Such studies have been reported by others (139, 144) and indicate that the *S* of the amino acyl RNA is indistinguishable from that of the free RNA. Thus, there is general agreement, with the exceptions noted, that each amino acid-

acceptor RNA chain is of the order of 75 to 100 nucleotides long. At present there is no information concerning how small the chain may be (assuming it has the correct terminal trinucleotide sequence) and still be active as an amino acid acceptor.

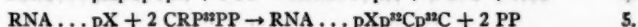
Based on changes in the optical density and specific rotation as a function of temperature (143, 144, 145, 147) and ionic strength (143, 147), it has been concluded that amino acid-acceptor RNA, like RNA of other types (148), contains regions of helical configuration resulting from intramolecular hydrogen-bonding between complementary bases (143, 144, 145, 147). From the magnitude of the hyperchromic transition (20 to 25 per cent), it is estimated that approximately 40 to 60 per cent of the bases are involved in hydrogen-bonding (143, 147, 148, 152). This degree of hydrogen-bonding very likely explains the resistance of amino acid-acceptor RNA to destruction by polynucleotide phosphorylase (149, 150) and venom diesterase (151). Judging from the relatively low temperature at which disruption of the secondary structure commences [the starting temperature is dependent on the ionic strength (143, 147, 148)] and the comparatively broad temperature range of the transition (45 to 50 degrees at 0.1 *M* salt), it does not seem likely that long continuous hydrogen-bonded helical regions exist (148, 152). A model has been proposed (152, 153) to rationalize the relatively high degree of hydrogen-bonding indicated by the magnitude of the hyperchromic shift, with the relatively few hydrogen bonds to be expected from a random sequence of nucleotides in the acceptor RNA (152). This involves "looping out" non-complementary base pairs, thereby allowing for a maximum number of adjacent hydrogen-bonded nucleotide pairs.

This "looping out" model raises the question of whether such loops provide for structural differentiation amongst chains which, in turn, can be related to their functional specificity (152). In this regard, amino acid-acceptor activity is only slowly inactivated by heating at 100° [(144, 154); see, however (145)], but this may be attributed to rapid reformation of the original hydrogen-bonded structure upon cooling (143, 144, 147, 154). More recent experiments suggest that amino acid-acceptor activity does not depend on the intactness of the secondary structure (145): although heating and rapid cooling of concentrated solutions of acceptor RNA produces (a) a doubling of the weight-average molecular weight, (b) an increased heterogeneity of RNA molecules within the population, and (c) a different extinction coefficient (all of which are believed to be attributable to intermolecular hydrogen-bond formation), there is no effect on the ability to bind amino acids.

Additional attempts to detect the basis of the functional heterogeneity amongst acceptor RNA chains have been concerned with studies of nucleotide composition and sequence. Analyses of the base composition of acceptor RNA's from a variety of sources show them to differ markedly from RNA of the ribonucleoprotein particles [(114, 140, 141, 144, 146; 154 to 157); see also (1)]. The most striking features of the composition are: (a) the equiva-

lence between the amount of 6-amino purine and 6-keto pyrimidine and between the 6-keto purine and 6-amino pyrimidine (see references above); and (b) the relatively high levels of "unusual" nucleotides (140, 144, 155, 156, 158). The base-equivalence, so characteristic of DNA, is very likely related to the intramolecular hydrogen-bonding. The "unusual" nucleotides detected so far [e.g., in *E. coli* (155)] include 5-ribosyluridylylate, ribothymidylylate, 2-methyladenylylate, 6-methyl amino purine nucleotide, and 1-methyl guanylate at levels of 2 to 4, 1, 0.2, 0.07, and 0.1 moles per 100 moles of nucleotide, respectively. Nucleotides containing 2-O-methyl ribose (159) and 5-methyl cytosine (160, 161) have also been detected in acceptor RNA. Since the methylated purines are present at levels of less than one per RNA chain, they cannot be essential for acceptor activity for every amino acid but perhaps only for certain ones. There is, however, a strong correlation between the concentration of 5-ribosyluridylylate and the ability of RNA to accept amino acids (156). The significance of this correlation in terms of the requirement of the nucleotide for amino acid-acceptor activity is now known.

The studies mentioned above, while establishing similarities amongst the acceptor RNA chains, give no indication of differences which might distinguish one chain from another. Current studies (162) have, however, demonstrated that there are differences in the sequence of nucleotides adjacent to the terminal trinucleotide. After pyrophosphorolysis of *E. coli* acceptor RNA (118), so that only the terminal three nucleotides from all chains had been removed (Reaction 4), the RNA was labeled with P^{32} by the incorporation of two equivalents of cytidylic acid- P^{32} per RNA chain (Reaction 5). Since the innermost P^{32} atom is linked to the 3'-hydroxyl group of the fourth nucleotide in the chain, alkaline hydrolysis and separation of



the labeled nucleotides permit one to calculate the proportion of acceptor RNA chains having one or another nucleotide in the fourth position. Thus 68, 24, 8, and <1 per cent of the chains in the population have adenylylate, guanylylate, uridylylate, and cytidylylate, respectively, in the fourth position. An alternative to alkaline hydrolysis was RNase digestion of the same P^{32} -labeled RNA preparation. This resulted in the conversion of the RNA into P^{32} -labeled fragments (mono-, di-, and trinucleotides) which were separated and identified by chromatographic and ionophoretic procedures. The various terminal nucleotide sequences in the RNA, deduced from the composition and nucleotide sequence in the isolated fragments, are summarized in Table I. By far the largest proportion of the RNA chains have adenylylate or guanylylate in the fourth position followed by a pyrimidine; in about 4 per cent of the chains, adenylylate in the fourth position is followed by guanylylate or another adenylylate. By use of still another technique, enzymatic hydrolysis with an

TABLE I

THE DISTRIBUTION OF TERMINAL TRINUCLEOTIDE SEQUENCES IN
AMINO ACID-ACCEPTOR RNA FROM *E. COLI*

Terminal nucleotide sequences identified*	Percentage of RNA chains having nucleotide sequence shown
... pPypApCpCpA	65.1
... pPypApApCpCpA	1.1
... pPypGpApCpCpA	3.1
... pPypGpCpCpA	17.1
... pPypApGpCpCpA	3.6
... pPypUpCpCpA	2.6
... pPypApUpCpCpA	1.4
† ... pPypRUpCpCpA	2.8
† ... pPypTpCpCpA	1.8

* The abbreviations used are standard in the biochemical and genetic literature. For simplicity in the designation of nucleotide sequences I have used the notation adopted by Markham & Smith (262). In this convention, the letters C, A, G, and U indicate cytosine, adenine, guanine, and uracil, respectively. Cp, Ap, Gp, and Up correspond to the 2'- or 3'-nucleoside monophosphates (or a mixture of both), while pC, pA, pG, and pU refer to the 5'-monophosphate derivatives. The designation ... pCpA denotes adenyllyl 5'→3' cytidilyl 5'→ - - - - . Py symbolizes pyrimidine.

† Preliminary identification of two of the mononucleotide fragments suggest the presence of 5-ribosyluridylylate and ribothymidylylate in the fourth position.

RNase prepared from Taka-diastrase (163), which specifically hydrolyzes linkages involving guanylic acid, chains having adenylate in the fourth position can be subdivided into at least 12 classes and the chains with guanylate as the fourth nucleotide into at least five types. This implies that the chains containing adenylate as the fourth nucleotide are heterogeneous with respect to the position of the first guanylate in the chain. Similarly, the RNA chains containing guanylate in the fourth position differ in the location of the next guanylate residue along the chain. The number of nucleotides and the sequences within each of the Taka-diastrase RNase-produced fragments are still under investigation. What is already clear from the alkaline and RNase digestions is that there are nucleotide differences amongst the population of acceptor RNA chains, which can now be examined with respect to their specificity as amino acid acceptors.

While it is clear that acceptor RNA is heterogeneous with respect to different amino acid-acceptor RNA chains, the possibility remains that there is also heterogeneity among the chains specific for a single amino acid. One indication of such inhomogeneity has been reported for the methionine-

acceptor RNA from *E. coli* (97), and a second one is suggested by the finding that preparations of *E. coli* acceptor RNA, when tested for leucine-acceptor activity with a leucyl RNA synthetase of mammalian liver origin, accepted less than one-third the amount of leucine than could be incorporated by a corresponding enzyme from *E. coli* (154). The extreme of this situation is the observation that acceptor RNA from nuclei of pig liver fails to accept alanine when tested with a cytoplasmic alanyl RNA synthetase but does so with the nuclear enzyme (164). On the other hand, the acceptor RNA from yeast accepts as much valine when tested with an ascites cell extract as it does with a yeast extract (140). Physical evidence of heterogeneity amongst RNA chains specific for a single amino acid is apparent from the bimodal peaks of tryptophan- and valine-acceptor RNA activity eluted from silicic acid columns (165). The most direct interpretation is that there is more than one type of RNA chain specific for each amino acid. It is conceivable that different chains carrying the same amino acid are utilized differently. It is important at this stage to obtain independent criteria for the homogeneity or heterogeneity of specific amino acid-acceptor RNA chains. Studies that detect quantitative differences in the amount of amino acid which can be bound to the RNA by different enzymes appear to be one way of examining this question.

Before concluding the discussion of specificity with regard to amino acyl RNA, it is worth commenting on present efforts to isolate specific amino acid-acceptor RNA chains, since such RNA preparations will very likely be required for extensive examination of the relationship between nucleotide sequences and amino acid specificity.

Approaches to this problem have utilized chromatography on cationic adsorbents (144, 166) and electrophoresis (99), but thus far these have only succeeded in separating groups of different amino acid-acceptor activities. Another attack uses countercurrent distribution techniques (167, 168), and this has led to the separation of several activities from each other. More recently, separation of acceptor activities has been reported using partition chromatography on silicic acid columns (165).

Other techniques which have been exploited depend on the selective alteration of either one, or all but one, specific class of RNA chains and the separation of the altered species from the rest. For example, marked enrichment for tyrosyl- and histidyl-RNA chains has been achieved by complexing them to polydiazostyrene columns (169, 170). An alternate technique employed periodate oxidation of all but one type of RNA chain and subsequent separation of the unoxidized amino acyl RNA derivative by fractional precipitation (171, 172). In each of these cases, eight- to twelvefold enrichment for the specific RNA was reported.

A recent finding indicates that the RNA chains specific for γ -glutamyl cysteine remain bound to the purified glutathione synthetase (173). If such enzyme-RNA combinations prove to be general for other amino acyl RNA

synthetases, then the most direct approach to the isolation of specific acceptor RNA chains may be to utilize the very enzyme which distinguishes one RNA from all others as the adsorbent or fractionating agent.

In summary, it appears from the studies of amino acyl RNA formation that the specificity of the reactions resides with the enzymes and their capacity to discriminate between closely related amino acid structures and to "recognize" an RNA chain that has a unique sequence of nucleotides. In each case, however, the amino acid is bound to an identical unit, a terminal adenylyl residue linked to a cytidylyl-cytidylyl sequence.

THE CONTRIBUTION OF RIBOSOMES TO THE SPECIFICITY OF PROTEIN SYNTHESIS

Evidence for the participation of ribonucleoprotein particles [microsomes and ribosomes (174)] in protein synthesis has been extensively reviewed elsewhere (1, 9, 11, 12, 14, 16) and will not be cited in this discussion. It has also been reported that isolated bacterial cell membranes [see (1, 2, 5, 9) for references and reviews of individual papers], mammalian cell nuclei (67, 175), and mitochondria (176, 177) incorporate amino acids into protein; net synthesis of cytochrome-*c* has been observed in mitochondrial preparations (176). It appears, however, that ribosomal particles may be the functional entities in these latter systems since the existence of ribosomes, amino acyl RNA synthetases, and acceptor RNA within lymphocytic nuclei (175, 178, 179) and mitochondria (178) has been demonstrated. In view of these uncertainties, the following discussion will be restricted to studies with partially purified ribosomal systems. Before examining the evidence regarding the postulated role of ribosomes in the specification of amino acid sequences, I will summarize some of the current data on the structure and composition of ribosomes.

The structure and composition of ribosomes.—The isolation of ribosomal particles from mammalian, plant, and microbial sources has been referred to and discussed in a number of recent publications (1, 14, 174, 180, 181) and need not be detailed here. In studies with bacteria, where it is known that the ratio of RNA to protein varies with the stage of growth and the growth rate (182, 183), the amount of ribosomes (14, 184) and perhaps the distribution in types of ribosomes (14, 185, 186, 187) also vary with the growth phase. Once extracted from the cell, the distribution of sizes of the ribosomes and their stability are a function of the ionic strength and the concentration of divalent cation, usually Mg^{++} [(14, 180); see also papers in (174)]. The polyamines, spermidine and putrescine, have also been observed to preserve the integrity of the ribosomes (188).

Ribosomes sediment in the ultracentrifuge as discrete, sharp boundaries with sedimentation coefficients dependent on the Mg^{++} concentration. At about 0.005 to 0.01 *M* Mg^{++} , large particles with an *S* of about 100 predominate, whereas at about 0.001 *M* Mg^{++} , depending on the source, the predominant ribosomal populations have an *S* of 70 to 80. With still lower levels

of Mg^{++} (10^{-4} to 10^{-5} M), or in the presence of divalent cation-binding agents particles of approximately 50 to 60 S and 30 to 40 S are present (14, 174, 180). Thus, by lowering the Mg^{++} concentration large ribosomes dissociate into subunits, and on raising the Mg^{++} concentration these subunits reassociate to form the large ribosomes (14, 174, 180). The mechanism whereby this reversible dissociation is controlled by divalent cations is not known. On the basis of the sedimentation and diffusion coefficients and the intrinsic viscosity, the following particle weights have been estimated [e.g., for *E. coli* ribosomes (180)]: 30 S, 1×10^6 ; 50 S, 1.8×10^6 ; 70 S, 3.1×10^6 ; and 100 S, 5.9×10^6 . The general conclusion derived from studies with ribosomes from *E. coli* (174, 180, 189) is as follows: $(100\text{ S}) \rightleftharpoons 2(70\text{ S}) \rightleftharpoons 2(50\text{ S}) + 2(30\text{ S})$; similar relationships exist for ribosomes obtained from other sources (174).

Earlier electron micrographs showed that the particles of 70 to 80 S derived from mammalian (see 190) and plant (191) sources are spherical with a diameter of about 150 to 200 Å. More recent photographs of *E. coli* ribosomes (192, 193) show that: particles of 30 S appear as irregularly shaped or prolate ellipsoids with average dimensions of 95×170 Å; particles of 50 S are nearly spherical with a diameter of about 140 to 180 Å; particles of 70 S appear as units of unequal size joined together to give irregularly shaped structures with a diameter and height of 200 and 170 Å respectively; and particles of 100 S appear as dimers of the 70 S particle with over-all dimensions of 350 to $400 \text{ Å} \times 140 \text{ Å}$. Although the microsomes contain lipid material (174), the ribosomes prepared from microsomes or isolated directly are composed entirely of RNA and protein (1, 14, 174, 180, 189). The presence of small quantities of other components, however, has not been rigorously excluded. The RNA content is generally 40 to 50 per cent in ribosomes from mammalian and plant sources (174), but 60 to 65 per cent in ribosomes from *E. coli* (180).

Phenol or detergent extraction of the RNA in ribosomes (194 to 199) indicate two components with sedimentation coefficients ranging from 15 to 18 S and 23 to 28 S. Together with diffusion coefficients or intrinsic viscosity values, the molecular weights calculated for the two RNA species vary between 5 to 6×10^5 and 1.1 to 1.3×10^6 , respectively. It seems clear (197) that the ribosomes of 30 S contain only the RNA component of 16 S while the particles of 50 and 70 S yield a mixture of the 16 S RNA and 25 S RNA. From the calculated molecular weights of the RNA and the particle weight of the 50 S ribosome it appears that a 50 S particle contains either one RNA molecule of 23 S or two RNA molecules of 16 S. Since the over-all nucleotide composition of the RNA from the ribosomes of 30 and 50 S is indistinguishable (200), it is possible that the larger component is composed of two small units of RNA (197). Upon heating the isolated RNA from liver ribosomes there is a fall in viscosity and the S drops to 8.2, yielding fractions with a molecular weight of 1.2×10^5 (194); these smaller RNA subunits have not been observed in bacterial preparations (197).

Ribosomal RNA in solution exhibits a reversible hyperchromic transition

on heating (147, 148, 201); this suggests the existence of intramolecular hydrogen-bonding between the bases. Since the degree of hypochromicity of the isolated RNA in solution and of the RNA within the ribosomes is the same, it has been concluded that the same extent of hydrogen-bonding exists in both (201).

Nucleotide analyses of ribosomal RNA from a variety of sources give similar values (1, 140, 144, 157, 200). Guanylic acid is invariably most abundant; uridylic and cytidylic acids, present in roughly equal amounts, are least abundant. 5-Ribosyl uridylic acid is present in only trace amounts, and there is little of any of the other "unusual" bases (157, 200, 202, 203).

Information concerning the structural proteins of ribosomes is scarce. Amino acid analyses of ribosomal proteins from rabbit reticulocytes and pea seedlings are strikingly similar (204). The proteins from ribosomes of 30 S, 50 S, 70 S, and 100 S, from *E. coli*, closely resemble each other; although there is marked heterogeneity amongst the proteins, there are two major N-terminal amino acids, alanine and methionine (205). In short, no outstanding differences have been detected amongst the ribosomal types of a given species or in those from different species.

The role of the ribosomes in the determination of amino acid sequences in protein.—According to the hypothesis outlined earlier, amino acyl polynucleotide derivatives are transferred to the ribosomes and become associated there through hydrogen bonds to a complementary sequence of nucleotides in the ribosomal RNA [the "adaptor hypothesis" (1, 25)]. It is not implied, however, that the region of the acceptor RNA reacting with the ribosomes is the same as the nucleotide sequence which specifies the binding of a particular amino acid to its corresponding acceptor-RNA chain. At present, the experimental evidence supporting the "adaptor hypothesis" is fragmentary.

Two recent experimental approaches have made possible an evaluation of the contribution of ribosomes to the specificity of protein synthesis. The first of these was the demonstration that amino acyl RNA is a more direct precursor for amino acid incorporation into the particles than free amino acids (206), and the second was the exploitation of a cell-free system from reticulocytes which synthesizes hemoglobin (207).

In the direct transfer from the amino acyl RNA, removal of the amino acid from the RNA at an extraribosomal stage does not seem to occur since there is little or no dilution of the incorporation of the unlabeled amino acid (11, 206). Transfer of the amino acid is stimulated, as is the incorporation of free amino acids, by the presence of ATP, GTP, and a non-dialyzable heat-labile fraction derived from the soluble portion of the tissue extract. By loading the reaction mixture with relatively large quantities of ribosomes and small amounts of amino acyl RNA, almost all of the amino acid can be incorporated into the ribosomes (11).

Some success has been achieved in purifying the enzyme which catalyzes the transfer of amino acid from RNA to the ribosomes. With sulphydryl

compounds to protect the transferring system from inactivation (208) and with ribosomes as acceptor, a partially purified enzyme was obtained which catalyzes the transfer of amino acyl groups to peptide linkage in the ribosomes (209). Additional studies (210) indicated that the ability to transfer various amino acids was purified to the same extent, suggesting, perhaps, the existence of a single transferring enzyme. Transfer of a given amino acid from acceptor RNA to the particle protein was increased if the other naturally occurring amino acids were also bound to the RNA (99); however, in other studies this was not observed (211). It has been reported that pea seedling ribosomes and acceptor RNA loaded with all the amino acids support net synthesis of protein and of ATPase activity (211). A relevant observation is that amino acids, linked to yeast or *E. coli* acceptor RNA, are transferred to mammalian ribosomes as well as from mammalian acceptor RNA (11, 99, 209). It is important, however, to determine whether such heterologous systems do in fact synthesize specific proteins (see later discussion).

Even with the above system, direct examination for the formation of complexes between the amino acyl RNA and the ribosomal RNA has not been possible. Thus far, the amino acids that become associated with the ribosomes are exclusively in peptide linkage. Thus, if complexes between the amino acyl RNA and the ribosomal RNA exist, they are either unstable or their steady state concentration is small.

Attempts to circumvent this obstacle have relied on measuring the uptake of the labeled RNA moiety (212, 213, 214). For example, it was found that the terminal AMP group of the RNA and bound valine became associated with the ribosomes in the same ratio as they existed in the precursor amino acyl RNA (11). Similarly, approximately 5 per cent of P^{32} -labeled acceptor RNA becomes bound to ribosomes, and this requires an energy-generating system (214). Based on the stability of the P^{32} in the ribosome to 8M urea, a covalent linkage between the acceptor RNA and the particles has been proposed (215). A more recent paper reports that P^{32} -labeled acceptor RNA, with or without bound amino acids, becomes rapidly associated with ribosomes (216). Of the P^{32} -RNA bound to the ribosomes, only 15 to 25 per cent is displaced in the presence of additional unlabeled acceptor RNA (216). The fate or state of the irreversibly-bound RNA is not known.

There is as yet no indication of how much of the acceptor RNA chain becomes associated with the ribosomes or whether the P^{32} -labeled material displaced from the particles is native acceptor RNA or some derivative of it. Attempts to detect an enzymatic modification of the amino acyl RNA prior to incorporation into the ribosomes, or as a result of incorporation, have been unsuccessful (216). There is, moreover, no information concerning the mode of association of the acceptor RNA with the ribosomes or whether the ribosomal RNA is involved in the reaction. Especially puzzling is the finding that free acceptor RNA is bound as effectively as the amino acyl derivative. It is quite possible, however, that the ribosome preparations still contain

sufficient amino acids and enzymes to form the amino acyl RNA (217).

Since rabbit reticulocytes synthesize principally hemoglobin (218), cell-free preparations from these cells have been useful for studies of the requirements for specific protein synthesis (207). Hemoglobin synthesis occurs in the presence of ribosomes, a "pH 5 precipitate" fraction from the supernatant fluid of reticulocyte lysates, GTP, an ATP-generating system, and a mixture of amino acids (207). Approximately 50 to 60 per cent of a labeled amino acid was recovered from hemoglobin present in the soluble phase of the reaction, and it was suggested that the remainder was ribosome-bound hemoglobin. The labeled amino acids were incorporated in the same ratio as they are known to exist in rabbit hemoglobin (207). With this system, the requirement for added amino acid-acceptor RNA for ribosomal hemoglobin synthesis was determined (219). It was found that, although there was significant incorporation of amino acids in the absence of added RNA, the incorporation was increased about twofold by RNA addition. Acceptor RNA from guinea pig liver was as effective as that isolated from reticulocytes. These experiments also suggested that acceptor RNA functions catalytically, since more amino acid was incorporated than could have been bound by the amount of acceptor RNA added.

An extension of these studies has led to the suggestion that the ribosomes contain incomplete peptide chains of hemoglobin and that amino acid incorporation in the cell-free system can only complete an already initiated peptide chain (220). Thus, very little, if any, incorporation of valine into the N-terminal positions of the two peptide chains of hemoglobin occurred under conditions in which valine was incorporated into internal positions. This system seems at present to be well-suited for investigating the requirements for initiation of new chains, the factors which effect dissociation of completed chains from the particles, and the mechanism for the final assembly of polypeptide units, in this case the two α - and two β -chains of hemoglobin.

The "adaptor hypothesis" assigns to the nucleotide sequences in ribosomal RNA the role of specifying the amino acid order in proteins (1, 25). One may ask whether amino acyl RNA compounds from any source interact with the ribosomal RNA in a specific way, i.e., to produce an amino acid sequence characteristic of the ribosomal code. Studies already cited (11, 99, 209) indicated that amino acid incorporation into protein does occur when amino acyl RNA and ribosomes from different sources are mixed. A test of the specificity of this incorporation has been made with the hemoglobin-synthesizing cell-free system from reticulocytes (221). Maximal formation of soluble hemoglobin was found to occur when reticulocyte ribosomes were supplemented with a "pH 5 precipitate" fraction (which contains the components necessary for forming the amino acyl RNA compounds), the "pH 5 soluble" fraction, and the necessary nucleoside triphosphates. By testing various combinations of ribosomes, "pH 5 precipitate," and "pH 5

supernatant" fractions from rabbit reticulocytes and guinea pig liver, the following results were found: (a) Reticulocyte ribosomes functioned equally well for amino acid incorporation and for the production of labeled hemoglobin when supplemented with the guinea pig liver "pH 5 precipitate" or with the reticulocyte "pH 5 precipitate" fraction. (b) Omission of the "pH 5 precipitate" fraction decreased the incorporation of amino acids by 60 to 80 per cent. (c) The amount of amino acid incorporation by reticulocyte ribosomes was unaffected by the presence or absence of the "pH 5 soluble" fraction; the recovery of the radioactivity as soluble hemoglobin, however, was increased by the reticulocyte "pH 5 soluble" fraction but not by the corresponding guinea pig component.

These data show that non-ribosomal fractions are required for maximal synthesis of hemoglobin and its release from the particles. Moreover, they demonstrate the existence of one or more factors in the "pH 5 supernatant" fraction which stimulate the removal of finished protein from the ribosomes. They are equivocal, however, with respect to interpretations of the contribution of the acceptor RNA. Equivalence of the two "pH 5 precipitate" fractions is not tantamount to an equivalence of the RNA components they contain. Without independent information on the amount of homologous acceptor RNA present in the particles, it is difficult to evaluate the extent to which the added heterologous acceptor RNA has entered into the incorporation reactions. It may be that the "pH 5 precipitate" fraction supplies only essential enzymes and that the added acceptor RNA is redundant.

An additional attempt (221) to test the prediction that amino acid sequences are determined solely by the ribosomes was carried out with fractions from sheep and rabbit reticulocytes, each of which produce chromatographically separable hemoglobins. These experiments showed that, when rabbit ribosomes, rabbit "pH 5 precipitate," and rabbit "pH 5 soluble" fractions were combined, all of the hemoglobin formed was of the rabbit type. When sheep "pH 5 precipitate" was substituted for the rabbit "pH 5 precipitate," essentially all of the soluble hemoglobin was still of the rabbit type. The use of all sheep fractions gave only sheep hemoglobin and substitution with rabbit "pH 5 precipitate" still yielded sheep hemoglobin. In these experiments, however, omission of the "pH 5 precipitate" fraction from the otherwise complete system had no effect on the incorporation of amino acids into ribosomal proteins or soluble hemoglobin. This implies, as pointed out above, that sufficient amino acid-acceptor RNA and the enzymes which form the amino acyl RNA compounds were present in the ribosomes, the "pH 5 supernatant" fraction, or both, to saturate the incorporation system, thereby minimizing any possible effect of the added heterologous acceptor RNA.

It was also reported that the mixture of sheep "pH 5 supernatant" and rabbit ribosomes resulted in the formation of significant amounts of both sheep and rabbit hemoglobins. Similarly, the addition of rabbit "pH 5 super-

nantant" to sheep ribosomes resulted in the formation of both types of hemoglobin.

This finding suggests that one or more soluble components, in addition to the ribosomes, may contribute to the determination of amino acid sequences in hemoglobin (221). It is possible, however, that the appearance of label in rabbit hemoglobin and sheep hemoglobin does not represent *de novo* synthesis of both hemoglobin types. It is not inconceivable, for example, that sheep hemoglobin present in the added sheep "pH 5 supernatant" fraction underwent dissociation and recombination with portions of newly synthesized rabbit hemoglobin during the incubation or subsequent analysis of the products. Dissociation and recombination of the α - and β -chains of hemoglobin to produce hybrid proteins has been observed with mutant human hemoglobin (222, 223) and between hemoglobins from different species (224). Studies of the labeling in the α - and β -chains of the two hemoglobins recovered from the incubations would resolve this issue.

EVIDENCE FOR AN ADDITIONAL RNA COMPONENT IN PROTEIN SYNTHESIS

There are a number of observations which are presently inconsistent with the idea that ribosomes and amino acid-acceptor RNA are the sole determinants of amino acid sequences in protein. It has already been pointed out that specific protein synthesis ceases following P^{32} decay within the controlling DNA (77, 85). Excluding DNA from a direct role in amino acid sequence specification (see earlier discussion), these findings imply that the protein-forming system is unstable and must be regenerated.

Participation of an unstable but essential component in protein synthesis is also suggested by the dependence of protein formation on concomitant RNA synthesis [see the following references for discussion and bibliographies to older papers on this subject (2, 9, 15, 16, 69)]. In recent years, studies with analogues of the naturally-occurring purines and pyrimidines have demonstrated marked inhibition of the formation of specific proteins but comparatively lesser effects on total protein synthesis (see 2, 9, 15). This has generally been interpreted as resulting from the formation of altered RNA templates which, although functional in polypeptide synthesis, yield modified inactive proteins. The concept of analogue-induced altered protein formation is further supported by the finding that azaguanine, which can replace guanine in RNA [see (268) for a recent summary of information on the incorporation of base analogues into nucleic acids], has a greater effect on the incorporation of the sulfur amino acids into protein than on that of other amino acids (225). Similarly, 5-fluorouracil, which is also incorporated into RNA (268), induces the formation of proteins in *E. coli* with a lowered content of proline and tyrosine and elevated levels of arginine (226). Even more striking is the finding that alkaline phosphatase and β -galactosidase, formed in the presence of 5-fluorouracil, are structurally altered (227). Since 5-fluorouracil and, presumably, 8-

azaguanine [(226, 227, 228); see also (2)] produce a relatively rapid effect on the structure of the proteins being formed and since the altered, but not the native, protein continues to be made, the existence of an unstable RNA component in protein synthesis seems likely (229).

Unfortunately, little information relevant to the stability of the ribosomes and acceptor RNA during protein formation is available. The apparent instability of ribosomes in *in vitro* systems may very well result from environmental features not existing in the cell (e.g., pH, ionic strength, divalent cation concentrations, degradative enzymes, etc.). The earlier investigations into RNA turnover in growing cells (230, 231, 232) and a more recent study (233) suggest that there is little or no degradation and resynthesis of the bulk of the cellular RNA during growth, and it is not unreasonable to extrapolate this conclusion to include intact ribosomes. It is not known, however, whether ribosomes undergo cyclic alterations between active and inactive states during protein synthesis.

There is also little information regarding acceptor RNA turnover. An enzyme has been described which catalyzes the removal and resynthesis of the terminal trinucleotide segment of acceptor RNA chains (115 to 118); however, its significance in protein synthesis is unknown. Turnover studies suggest that the "soluble" RNA fraction (which includes the amino acid-acceptor RNA) turns over more rapidly than the particulate RNA (231, 234). However, breakdown and resynthesis of acceptor RNA do not appear to occur during the incorporation of leucine into reticulocyte ribosomal protein, since the amount of leucine incorporated was greater than the amount which could be linked to the acceptor RNA present (219).

Experiments with growing cells have failed to detect significant turnover of RNA (230, 231, 232). However, it was noted several years ago that, although net synthesis of RNA ceases following infection of *E. coli* with T-bacteriophages (235), P^{32} -inorganic phosphate added at or soon after infection is rapidly assimilated into cellular RNA (236). Upon analysis of this P^{32} incorporation, the following major facts have emerged (237, 238): (a) P^{32} -phosphate added within 10 min. after phage infection is rapidly incorporated into the cellular RNA; the rate of P^{32} incorporation into RNA is, in the earliest times following infection, faster than into DNA. (b) Addition of unlabeled phosphate, or removal of the P^{32} several minutes after the addition of the label, results in a rapid loss of between 50 to 70 per cent of the incorporated P^{32} . This is in contrast to the situation with uninfected cells, where P^{32} is not displaced from newly formed RNA after removal of the label. (c) The base composition of the newly synthesized RNA, estimated from the proportions of the total radioactivity contained in the 2'-3'-nucleoside monophosphates after alkaline hydrolysis, resembles the base composition of the infecting phage DNA. The fraction of the RNA which does not turn over (i.e., that which remains after removal of the label) has a base composition resembling normal *E. coli* RNA.

A clue to the function of the phage-induced RNA formation was the finding that protein synthesis must precede replication of the infecting DNA (239, 240, 241). Part, but not all (242), of the requirement for early protein synthesis is explained by the synthesis of hydroxymethylase (78), hydroxymethyldeoxycytidylate (79, 80, 81), deoxguanylate (79, 81) and deoxythymidylate (79, 81) kinases, deoxycytidylate deaminase (83), deoxycytidine triphosphatase (79, 82), hydroxymethylcytidylate-DNA glucosylases (79, 243), and DNA-polymerase (244). The close correlation in time between the synthesis of new RNA and phage-specific proteins suggests that the two processes may be related (245).

Studies of the phage-induced RNA revealed that about 60 per cent is associated with free ribosomes, 30 per cent with ribosomes bound to cell membranes and large cell fragments, and 8 per cent with a non-sedimentable fraction (246). The RNA bound to free ribosomes dissociates at low Mg^{++} concentrations to yield free RNA with an S of about 8 (246); this distinguishes it from the RNA components of 18S and 25S from uninfected *E. coli* ribosomes (194, 195, 196). The non-sedimentable RNA has an S of 4 and, except for the altered base composition, resembles normal acceptor RNA from *E. coli* (246).

Studies of the base composition of DNA and RNA in a variety of bacterial species (247) have suggested the existence of an RNA component with a base ratio resembling that of the DNA. Recently, an RNA fraction with a high rate of turnover and a composition resembling the DNA has been detected in growing yeast (248).

A further clue as to the nature and function of the phage-induced RNA comes from the recent observation that the isolated phage-induced RNA (246) forms a hybrid complex with DNA from the infecting phage but not with other DNA (249). When a mixture of P^{22} phage-induced RNA and heated T2-DNA was cooled slowly (53), a new component, which sedimented like DNA in a cesium chloride-density gradient, was observed (249). It was suggested that the newly formed complex was a double-stranded DNA-RNA hybrid and that it resulted from hydrogen-bond formation between complementary nucleotide sequences in the two polynucleotides.

The existence of a rapidly turning-over RNA component is consistent with the long-recognized dependence of protein synthesis on continued RNA formation. It is also consistent with the inference that the incorporation of 5-fluorouracil and 8-azaguanine into RNA leads to a rapid formation of structurally altered proteins (227, 229). The additional finding that relatively few, if any, new ribosomes are formed by *E. coli* cells following phage infection (250) suggests that this rapidly turning-over RNA, and not the ribosomes, may be the determinant of amino acid sequences in the assembly mechanism of protein synthesis.

Several features of the phage-induced RNA formation, as it relates to the above hypothesis, should be commented upon. Following phage infec-

tion of *E. coli*, P^{32} -phosphate is incorporated into a relatively "stable," as well as the "unstable," RNA fraction. This "stable" component, which can account for as much as 50 per cent of the newly formed RNA, has a base composition different from that of the infecting phage; it more closely resembles the composition of the bacterial host RNA (237). Since it is the average base composition of the newly synthesized RNA which has been measured (237, 238), it would appear that the rapidly turning-over RNA has a base composition different from the average composition of the infecting phage DNA. It is not inconceivable that the base compositions of the "unstable" and "stable" RNA reflect the composition of different regions of the phage DNA; the "unstable" RNA may correspond to regions of DNA that contain higher than average amounts of adenine and thymine, whereas the more "stable" fraction reflects regions of lower than average adenine and thymine content.

The significance of the "stable" RNA fraction is not clear. The ratio of P^{32} -phosphate incorporated into the "stable" and "unstable" RNA fractions increases throughout the latent period (238). Moreover, the rate of P^{32} -phosphate incorporation into RNA decreases markedly later in the latent period (238). Nevertheless, protein synthesis continues at an almost constant rate up to the time of cell lysis (251). There are indications that the synthesis of proteins later in the latent period is not associated with an RNA fraction that turns over rapidly. 6-Azauracil, which blocks RNA synthesis, inhibits phage production only when added up to approximately 5 min. after phage infection. Thus, phage-protein synthesis can occur in the absence of RNA synthesis, provided that RNA synthesis is allowed to occur for a certain period after phage infection (245, 252).

CONCLUDING REMARKS

There are several indications that amino acid incorporation into peptide linkage can occur by a mechanism that does not involve amino acyl RNA synthetases. With a large-particle fraction from *Alkaligenes faecalis*, amino acid incorporation was dependent on a soluble protein; this protein has been purified from *Azotobacter vinlandii* and found to be devoid of amino acyl RNA synthetase activity when tested by ATP-PP exchange (253). Nevertheless, it was capable of replacing the mammalian "pH 5 precipitate" fraction for incorporation of amino acids into mammalian ribosomes (254). A similar result with a purified protein from a mammalian source [the "S protein" (255)] has been reported (256). Since well-washed mammalian ribosomes contain detectable quantities of amino acyl RNA synthetases (217) and acceptor RNA (257), the precise function of this protein remains obscure.

Further studies with the bacterial "incorporation enzyme" (253) have shown that it catalyzes an amino acid- and RNA-independent exchange of all four nucleoside diphosphates with the corresponding nucleoside triphos-

phates (258). In the presence of amino acids, each of the four nucleoside triphosphates is cleaved to inorganic phosphate and the nucleoside diphosphates (259, 260). Of considerable interest is the finding that each nucleoside triphosphate reacts with only a specific set of amino acids (259, 260). The nature of the phosphate elimination and the basis for the specificity between amino acids and nucleoside triphosphates is not clear. It has been reported, however, that, in the presence of a mixture of amino acids and a nucleoside triphosphate, peptides are formed concomitantly with the liberation of inorganic phosphate (259, 260). The mechanism of peptide formation and its significance to protein synthesis are still under study (259, 260). It may be that these reactions are related to the finding of nucleoside monophosphate-bound peptides and the ATP-dependent formation of peptide hydroxamates (261).

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PROTEIN NUTRITION^{1,2}

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INTRODUCTION

No other recent single aspect of nutrition has so stimulated international research as has protein malnutrition (kwashiorkor). The appalling toll of this disease among helpless infants and preschool children throughout most of the world and its frequent association with the blinding effects of avitaminosis-A, coupled with the contrast of its absence from the technologically advanced countries, have all contributed to the fascination which it holds for nutritional scientists. Its widespread occurrence among emerging nations and the recognition that there exists a technological potential for eradication of the syndrome have directed the concerted interests of the United Nations' agencies, of the Committee on Protein Malnutrition of the Food and Nutrition Board, the Commission for Technical Co-operation in Africa South of the Sahara, and other groups toward solution of this problem.

Restrictions of space have prohibited us from including all of the hundreds of publications reviewed for preparation of this manuscript. Perforce we have selected for discussion those studies which exemplify developments during the period covered. By this selection we imply neither exclusion on the basis of lack of scientific merit nor inclusion on the basis of priority. We apologize for omission of many excellent publications.

Continuing major attention has been given to further understanding of kwashiorkor, to a quantitative definition of protein and amino acid requirements of infants and adults, and to clarification of amino acid imbalance. Limitations of existing methodology for the nutritional evaluation of proteins have been recognized, and efforts to improve procedures are receiving attention. A major prejudice against vegetable proteins and protein concentrates for human and infant feeding has been broken down. Milk has been removed from its "holy" position as the source of protein for infants and small children; the relative merits for infant feeding of human versus animal milk have been objectively investigated, and restimulation of research on human milk has occurred. Finally, an emergence of nutritional chemistry is seen as a major consideration in technological processing of potential protein sources. The pure biochemist may wonder that some of these aspects are included in this volume, but to be convinced he need only observe the effects—physical, emotional, and political—of protein deprivation and then reflect upon the ultimate application of his pure biochemical knowledge.

¹ The survey of literature pertaining to this review was concluded in September, 1960.

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PROTEIN REQUIREMENTS

Evidence regarding protein requirements continues to suggest that the current protein allowances of the National Research Council are liberal. Hegsted (1) based an estimate of the minimum protein requirement of children on the following assumptions: (a) basal allowance estimated by the formula of Terroine (2); (b) growth allowance derived from growth curves; and (c) approximation of fecal losses. The Committee on Protein Requirements of the Food and Agriculture Organization (FAO) apparently based their estimates of requirements on similar considerations (3). In order to adjust for individual variation in requirements, they recommend that, for practical application, there be used a 50 per cent increase in the case of adults and a 25 to 30 per cent increase in the case of infants. In addition, a second correction factor is proposed to deal with increased needs of individuals on diets containing poor quality protein. Current disagreement over protein need in early life is indicated by the absence from the 1958 revision of the Food and Nutrition Board's Allowances of an allowance for infants (4). In 1955 the Board recommended 3.5 gm/kg body weight. The latter figure was subject to the criticism that it could not be met by breast feeding. Hegsted's minimal requirement of 2.5 gm/kg during the first few months of life has similarly been criticized, but he (1) contends that the data of Fomon & May (5) demonstrate that the protein supplied by breast milk will meet his theoretical estimates.

Studies on rats (6) fail to reveal significant differences for this species between cows' milk and human milk in the protein efficiency ratio, protein retention efficiency, net protein utilization, biological value, and true digestibility. A similar absence of differences was reported in infants who presumably were not protein deficient (7, 8). Waterlow *et al.* (9) found that 17 malnourished infants retained an average of 49 per cent of the nitrogen of human milk as opposed to 41 per cent of the nitrogen of cows' milk. They suggest that this small difference might be of biological significance for infants living on marginal protein intakes. The data of Fomon & May (5) further substantiate the observation that, as nitrogen intake is increased, the body seems to retain more nitrogen. Snyderman *et al.* (10), in a similar observation on premature infants, found that the increased retention could not be fully accounted for by the errors in the balance technique outlined by Wallace (11), and conclude that the additional nitrogen retention increased "chemical maturation." Cusworth *et al.* (12) have suggested that at least part of the retained nitrogen may be in the form of free amino acids rather than in body protein.

Although it has been generally believed that premature infants require more nitrogen for growth than full-term infants, Holt *et al.* (13) consider the minimum daily protein requirement of the premature infant, weighing 1400 gm. or more, to be around 2 gm/kg. Not all infants with birth weights as low as 1200 gm. gain normally on such intakes. Borgstrom *et al.* (14) demonstrated that protein absorption in the premature is as efficient as in the full-

term infant, despite a reduced content of trypsin. Malnourished Jamaican infants responded to only 2 gm. of protein/kg. body weight/day from either human or cows' milk, and it was suggested (15) that this level should be adequate for normal growth and maintenance. Similar studies (16, 17) of the growth of underprivileged Indian infants also suggest that the daily protein requirement during infancy approximates 2 gm. of protein/kg. body weight.

In a sorghum and millet-consuming region in Nigeria, Nicol (18) found the protein consumption of adults was 1.5 to 2.5 times the "safe practical allowance" calculated by the FAO (3), but in a yam-consuming area the protein consumption barely met the "minimum requirements" and never equalled the "safe practical allowances." There was no obvious clinical protein malnutrition, anemia, or reduced plasma protein levels in individuals over nine years of age in either area, and Nicol concluded that the "safe practical protein allowances" of the FAO are unnecessarily high. The Committee of National Nutrition and Resources Council of Japan (19) has provisionally adopted 0.5 gm/kg/day as the average minimum adult requirement for the type of protein supplied in the usual Japanese diet. A "safe practical allowance" of 1.25 gm/kg body weight is currently recommended for Japanese adults.

Clark *et al.* (20) confirmed that total caloric intake influences nitrogen balance in adult human subjects fed diets containing cereals plus amino acid supplements. Swift *et al.* (21) found no appreciable difference in the energy utilization by humans on isocaloric and isonitrogenous diets which contained widely different amounts of fat. There were, however, large caloric gains and increased nitrogen retention without accompanying weight gains on the high-fat (34.5 per cent) diets. Swendseid *et al.* (22) studied the utilization of the non-essential nitrogen sources (glycine, urea, diammonium citrate, and a mixture of non-essential amino acids) fed as supplements to diets that contained minimum amounts of the essential amino acids of egg protein. Glycine was not as well-utilized as either the mixture of non-essential amino acids or of glycine and diammonium citrate.

Munro *et al.* (23) conclude that muscle is the primary site of the protein-sparing action of carbohydrate in the rat, since the administration of glucose by stomach tube one-half hour after intraperitoneal injection of a labeled amino acid caused an increased deposition of radioactive amino acids in muscle protein with little deposition in the liver. Different types of dietary fat did not affect nitrogen balance in the rat (24, 25). Rats which were forced-fed at spaced intervals synthesized 10 to 20 per cent less body protein and showed a 50 to 100 per cent increase in body fat over pair-fed rats permitted to feed *ad libitum* (26).

AMINO ACID REQUIREMENTS

Since protein requirements vary with protein quality and since this variation reflects amino acid composition, the FAO (3) proposed as a new standard of reference a theoretical protein with an amino acid pattern based on the

minimum essential amino acid requirement of man. This pattern differs but slightly from the patterns of the other commonly used reference proteins (cows' milk, whole egg protein). Publication of this FAO reference pattern has greatly stimulated research designed to evaluate its applicability. If the proportions of individual essential amino acids are optimal for human growth and development according to the FAO reference patterns, human diets could theoretically be devised so that the amino acid pattern coincides with that of the reference. Since the availability of amino acids in a diet does not necessarily coincide with its amino acid content and the protein requirement is not the same in all physiological states, human and animal studies have been made to test the FAO reference patterns.

Workers at the Institute of Nutrition for Central America and Panama (INCAP) used the FAO reference patterns in designing diets to be fed to young children. Bressani *et al.* (27) fed maize protein at a level of 3 gm/kg body weight plus synthetic essential amino acids to match the amino acid pattern of the FAO reference pattern. Nitrogen retention was progressively increased by stepwise addition of tryptophan, lysine, and isoleucine. Nitrogen retention was depressed by the addition of sufficient methionine to bring the amino acid pattern of maize protein in line with that of the reference standard. This depression was reversed by further supplementation with isoleucine. A similar amino acid supplementation study (28) at levels of 1.5 and 2 gm. of protein/kg. body weight/day revealed that neither tryptophan nor lysine alone restored the negative nitrogen balance which occurred on the maize-based diet, but the addition of both amino acids resulted in an increased nitrogen retention. Again, nitrogen retention was further improved by addition of isoleucine but decreased by addition of methionine. The consistent adverse effect of methionine is interpreted by these authors to indicate that the FAO reference pattern contains too much methionine in proportion to the other amino acids. In another study (29), supplementation of wheat protein diets fed at a level of 2 gm. protein/kg/day with appropriate amounts of lysine, tryptophan, methionine, isoleucine, and threonine to conform to the FAO reference pattern resulted in improved nitrogen balance with the major part of the increased nitrogen retention being brought about by the addition of lysine alone. Addition of methionine did not alter nitrogen balance, and the workers again conclude that the methionine content of the FAO reference pattern is too high. Supplementation of wheat protein with a mixture of amino acids that contained no lysine resulted either in refusal of the food offered, or vomiting, or both. This is reminiscent of the findings of Kumta (30) that rats refused imbalanced diets almost immediately. Certain results of the Central America-Panama group (INCAP) with children have been confirmed in adults by Truswell & Brock (31), who report improved nitrogen retention upon the addition of lysine and tryptophan to maize diets.

Howe (32) found that the FAO reference pattern promoted somewhat greater growth in rats than did a similar mixture patterned after the essential amino acid content of casein, provided the percentage of essential amino

acids in the two mixtures was the same. FAO reference patterns supported more rapid growth when the diets were fed *ad libitum*, but the protein efficiency ratio of the FAO and casein patterns were equal at a lower essential amino acid content. In the case of the reference pattern of the FAO, the protein efficiency ratio was not changed if the tryptophan content was decreased by 33 to 50 per cent or if the lysine content was increased by 22 per cent. If casein was supplemented to the composition of the FAO reference pattern by the addition of amino acids, the resulting product was nutritionally equivalent to the FAO reference amino acid mixture, but a cottonseed flour-based product was inferior. This suggests that casein is digested in such a way that utilization of the amino acids is the same whether they are fed free or combined as part of casein but that this is not the case with cottonseed flour. Thus, a suspected theoretical flaw in sole use of the FAO pattern to appraise proteins has been demonstrated experimentally.

Snyderman *et al.* (33) found, in feeding studies with infants, that there is no justification for the supplementation of cows' milk mixtures with lysine. The estimated requirement of lysine in mg/kg/day was 88 to 90 for four infants and 102 and 101 for two others. The requirements of two premature infants for valine were found (34) to be 85 and 105 mg/kg/day, whereas two full-term infants required 85 and 87, and a third, 105 mg. Three infants, aged one and one-half weeks to three and one-half months, gained weight well, retained adequate nitrogen, and appeared in excellent health when arginine was omitted from the synthetic diet for periods of from 14 to 35 days (35). Auricchio *et al.* (36) examined the urines of newborn children from the first to the thirtieth day of life and concluded that during the first two weeks little or no conversion of tryptophan to niacin takes place. This is in accord with previous findings in the infant rat, guinea pig, and rabbit (37).

Nakagawa *et al.* (38) fed the minimal amino acid mixture of Rose (39) to 13 to 14 year old boys and found that they needed two to three times the minimal amounts of essential amino acid mixture required by Rose's adults. Since these boys required a daily minimum of 1.5 to 2.0 gm. of protein/kg. body weight to remain in nitrogen equilibrium—an amount far in excess of most recommended levels of intake—it may be questioned whether the experimental periods were sufficiently long to permit true equilibrium to be attained.

Clark *et al.* (40) found that five men needed a minimum of 400 to 1200 mg. of lysine per day to maintain nitrogen balance, and five women needed 300 to 700 mg. per day when fed diets consisting of wheat flour, maize, and amino acids. These results agree reasonably well with those reported by Rose and Levertan in spite of the fact that the diets used were quite different. Swendseid *et al.* (41) found no relationship between the amino acid requirements of young men and women at two levels of nitrogen intake, even though under similar circumstances Tuttle *et al.* (42) observed a definite increase in requirements of older men. Watts *et al.* (43, 44, 45) report that essential amino acids from egg, pork, milk, milk plus egg supplement, cottage cheese,

and beef muscle are about 90 per cent available to the human. Amino acid availabilities from peanut butter were lower with methionine being only 74 per cent available.

AMINO ACID REQUIREMENTS OF ANIMALS

Rama Rao *et al.* (46) redetermined the amino acid needs of weanling rats using casein-amino acid mixtures at a 10 per cent protein level. Under these conditions, requirements were (as per cent of diet) lysine, 0.9; histidine, 0.21; tryptophan, 0.11; isoleucine, 0.55; leucine, 0.69; valine, 0.56; threonine, 0.51; methionine plus cystine, 0.49; and phenylalanine plus tyrosine, 0.72. All of these values are somewhat lower than those of Rose *et al.* (47), except for threonine and isoleucine. In a further study of the optimum protein requirement for growth and nitrogen retention (48), the minimum requirement of protein for maximum growth (8.8 per cent of the diet) did not coincide with that required for maximum nitrogen retention (10 per cent of total diet).

Metta *et al.* (49) were unable to extend to rats the observation of Rose *et al.* (50) that more calories are required by humans to maintain nitrogen equilibrium on diets containing amino acids than on diets containing an isonitrogenous protein source. Rama Rao *et al.* (51) report that the addition of 50 per cent lard to diets that contain purified amino acids resulted in growth failure and a diseased appearance; these were not corrected by extradietary B vitamins. The syndrome did not occur when egg protein replaced the amino acids. Gerulat & Berg (52) found that excess dietary DL-leucine inhibited inversion of D-valine in the rat and that 2 per cent of DL-norleucine retarded growth on diets that contained either D-valine or D-leucine. Reid & von Sallmann (53) found that the tryptophan requirement of the guinea pig for normal development of the eye is greater than that for maximum growth. D-Tryptophan was only one-third to one-fourth as effective as the L- form.

Forbes & Rao (54) noted for rats that the lysine requirement, expressed as a percentage of the protein requirement, decreased progressively with age; i.e., the lysine requirement of a rat weighing 60 gm. was 5.98 per cent and of a rat weighing 445 gm., 0.77 per cent. The tryptophan requirement similarly decreased from 0.8 to 0.3 per cent.

Birnbaum *et al.* (55) continued their studies of water-soluble chemically defined diets. In mice, the use of a non-essential amino acid supplement composed of L-tyrosine, L-glutamic acid, L-proline, L-alanine, glycine, L-serine, L-cysteine, and L-aspartic acid supported weight gains similar to those obtained with Purina laboratory chow, whereas diets containing a supplement of the first four non-essential amino acids permitted but poor growth; rats behaved similarly. Leveille & Fisher (56, 57, 58) determined the quantitative amino acid requirements for maintenance of nitrogen retention (the lowest level of amino acid that maintains the nitrogen retention observed with the complete starting diet) and the minimum maintenance level (the lowest concentration that maintains nitrogen equilibrium) for adult roosters. A highly significant correlation exists between the maintenance requirement and the

amino acid content of feather protein. Klain *et al.* (59), using crystalline amino acids, studied the nitrogen requirement of growing chicks. For optimum efficiency of food utilization, 4 per cent nitrogen was required. At lower protein intakes, carcass fat was increased, probably because chicks eat more on low-nitrogen diets and convert to body fat the excess calories accumulated in this process. The fact that the previous protein reserves of the experimental subject are of extreme importance when interpreting nitrogen balance data has been re-emphasized (60).

Ousterhout (61) studied single amino acid deficiencies in chicks and found that chicks fed diets deficient in valine or isoleucine succumb within three weeks, whereas those suffering from lysine or histidine deficiencies live the longest. It is suggested that the former die rapidly because of the induction of a severe amino acid imbalance involving leucine, whereas the latter are able to draw on less critical body stores for the resynthesis of more crucial protein.

It is not possible to detail all the literature that concerns the nutrient requirements of tissue cultures. It is of interest that Eagle (62) has grown human cells in a protein-free, chemically defined growth medium equilibrated across a cellophane membrane with a medium containing 1 to 5 per cent dialyzed serum and a dialyzed pancreatic extract. The inference is that serum provides essential growth factors of small molecular weight, which are either initially bound to serum protein or formed from it by proteolysis. Also, Neuman & Lytell (63) have demonstrated that chicken embryo and maturing cockerel cultures require the 13 amino acids shown to be needed by mammalian cells, plus dialyzed chicken embryo extract and bovine serum.

AMINO ACID DEFICIENCIES

Sidransky and co-workers (64 to 67) studied the pathology of acute amino acid deficiencies in rats. Animals fed amino acid-deficient diets *ad libitum* exhibited few specific pathological changes, but force-feeding of purified diets devoid of methionine, valine, threonine, or lysine regularly induced pathological changes, particularly in the liver. No evidence of a general inhibition of protein synthesis in the liver was observed. Since the lesions were found to resemble some of those of kwashiorkor, diets that contained poor quality protein sources (maize, rice, wheat, milo, cassava) were also force-fed, and similar pathological changes resulted (68). Adams *et al.* (69) also produced a pathological condition resembling kwashiorkor by feeding rodents, *ad libitum*, a cassava diet that was low in protein and high in carbohydrate, and Volk & Lazarus (70) produced severe pancreatic acinar atrophy in the rabbit by feeding a low-protein diet.

AMINO ACID IMBALANCE

Attention continues to be focused upon amino acid imbalance. Harper (71) showed that the extent of growth retardation by an imbalanced mixture is related to the level of dietary protein, retardation being least at extremes

of protein intake. The same amount of the limiting amino acid completely corrected the growth depression regardless of the level of dietary protein. He suggested that a given amino acid imbalance increases the requirement of the limiting amino acid by a constant amount regardless of the dietary protein level. The studies imply that amino acid imbalance may not be of practical import in cases of either high or low protein intake.

Kumta & Harper (72) measured the magnitude of the increased need for histidine on imbalanced fibrin diets and also determined the order in which indispensable and dispensable amino acids become limiting for the rat when fed a low-fibrin diet (73). Morrison & Harper (74) report that only threonine can imbalance diets that contain 8 per cent casein plus cystine or DL-methionine. This contrasts with an earlier study in which phenylalanine was also reported to be effective. Although no direct evidence exists, it has been widely considered that imbalance is associated with impaired utilization of the limiting amino acid (75, 76) and that this results in an increase in the need for this amino acid. This hypothesis has been questioned by Fisher *et al.* (77), who found no evidence of increased lysine catabolism in chickens fed lysine-deficient imbalanced diets. These authors consider that the key to the growth depression may lie in finding an explanation for the reduced food intake that is an integral part of the imbalance picture. In another study bearing on this question, Rosenberg *et al.* (78) fed rice diets, supplemented with graded amounts of lysine and threonine, and found that for optimal performance the supplemented diet should contain 1.4 times as much lysine as threonine. As long as one of the two limiting amino acids was present in a suboptimal amount, growth and efficiency of food utilization were slightly improved by the addition of a small quantity of the other amino acid beyond the amount required for this balance. They ascribe this phenomenon to the law of mass action. Fisher, Shapiro & Griminger (79) have further noted that the high arginine requirement of chicks fed casein diets may be explained in part by the relative imbalance of this protein in regard to arginine.

PROTEIN METABOLISM

Crane & Neuberger (80) studied the digestion and absorption of protein by normal man. They fed whole and enzymatic digests of N^{15} -labeled yeast protein and found that about 28 per cent of the labeled nitrogen was excreted in the first 72 hours regardless of the form of the protein fed. This is somewhat less than the excretion of labeled glycine reported by Wu & Bishop (81). Blood and urinary urea were labeled within 20 to 30 min. after ingestion; the values at 20 min. were lower for the whole proteins than for the hydrolysate. This suggests that the time required for enzymatic hydrolysis may delay absorption only slightly. These results agree with observations of Gupta *et al.* (82), but the bearing this has on the rate of protein absorption under normal conditions cannot be stated since the amount of protein fed was very small (less than 3 gm.) and it was not fed with carbohydrates or fat. It has been shown, for example, that the presence of carbohydrates or fat in

the diet has a profound effect upon the rate of gastric emptying (83, 84). Holmes & Darke (85) observed that 43 adult African males who consumed indigenous diets absorbed from 70 to 89 per cent of the dietary nitrogen, whereas individuals who consumed control European-type diets absorbed 88 to 90 per cent. In this study mild hookworm infestation did not influence absorption, but in a later study nitrogen absorption was diminished by a heavy hookworm load (86).

The study of the cutaneous loss of nitrogen compounds by Darke (87) is of particular interest. Twelve adult Africans lost an average of 254 mg. \pm 22.9 mg. per day under conditions that did not induce visible sweating. The authors conclude that any nitrogen balance study which claims to measure output more closely than \pm 400 mg/day, even in the absence of visible sweating, would be invalidated unless cutaneous losses were included. This and other errors inherent in calculating nitrogen balance have also been considered by Wallace (11).

Kean (88) considers that the failure of protein-depleted rats rapidly to incorporate S^{35} into liver protein is because proteins with high turnover rates are not maintained on a low-protein diet. Dioguardi & Secchi (89) found that a low-protein diet caused a marked diminution in the nitrogen content of a single mitochondrion without a corresponding change of enzymatic activities, but fasting caused both to decrease. The authors propose that mitochondria contain two nitrogen-rich fractions: one is enzymatically inert, and the other contains the enzymes. Erwin (90) observed that in rats significant differences in the serum lipoprotein fractions, in some cases, could be attributed to the type of dietary protein. Madden (91) determined the albumin pool size of three hypoproteinemic patients before and after an intense nitrogen build-up and found no significant change.

Hypoproteinemia resulting from loss of serum proteins into the small intestine has been reported (92, 93, 94). Armstrong *et al.* (95) injected rabbits with I^{131} -labeled albumin and found what appeared to be labeled albumin in the lumen of the intestine. They calculated that the enzymatic breakdown of serum albumin in the gut can account for most of the breakdown *in vivo* and suggest that the gut may be the main site of degradation of albumin. Contrary to the findings of others (96), they could not demonstrate a significant breakdown of C^{14} -labeled albumin in any rat tissue-slice or homogenate system with the exception of the spleen (97).

Sobel *et al.* have reported on the changes in individual protein fractions in the rat heart during growth (98) and following starvation or cortisone administration (99). In protein depletion they found that individual proteins in the heart respond in a disproportionate manner, not necessarily related to the rate of accrual during normal growth.

BIOLOGICAL VALUE OF FOODSTUFFS AND DIETS

Emphasis on the eradication of protein malnutrition has revived interest in techniques of evaluating the nutritional value of proteins. Those concerned

with the practical problem of protein evaluation are keenly aware of the need for a rapid precise method for determination of the nutritional value of food proteins. Such a method should be simple and inexpensive for widespread application (100, 101). Collaborative studies of the protein efficiency ratio method (102) have led to its tentative adoption by the Association of Official Agricultural Chemists for material containing in excess of 9 per cent protein. Campbell and co-workers (103, 104, 105) evaluated the factors that influence values obtained with this technique and conclude that it can be used to advantage if a carefully standardized procedure is employed. Campbell (106) also noted that the protein value of cereal diets is usually limited by lysine, whereas meat products are usually limited by cystine or cystine plus methionine. He has devised a simplified chemical score based on comparison of lysine or methionine-cystine content of the foodstuff to that of whole egg protein. Such chemical scores correlated well with the protein efficiency ratios determined for 43 foodstuffs and were also closely related to the protein efficiency ratios found for foods limited in other amino acids. By multiplying the protein efficiency ratio by the grams of protein in a "reasonable daily intake of the foodstuff" a "protein rating" was obtained. This rating is currently used unofficially for evaluating the protein content of foods sold in Canada.

Rippon (107) compared six methods of estimating the nutritive value of protein and concluded that the nitrogen balance technique is the most reliable if nitrogen intake is constant in all tests and if urinary and fecal metabolic nitrogen of the test animal is determined before each test. Protein efficiency ratios determined by a modification of the depletion-repletion method (108) correlated well with the biological values. In general, the chemical scores agreed with the nitrogen studies. Bender (109) re-evaluated the chemical score procedure of Block & Mitchell (110) in light of newer knowledge of amino acid composition of foods and of amino acid requirements of the rat and found that when the biological values fell between 40 and 100 the chemical scores were numerically the same. If the biological value of a mixture fell below 30, the chemical score varied, depending upon the limiting amino acid. Mixtures devoid of lysine (i.e., chemical score = 0) have a biological value of 30; those devoid of leucine, histidine, isoleucine, tryptophan, or phenylalanine have a biological value equal to 20; and those lacking in threonine or cystine and methionine have no biological value. Hughes (111) compared the amino acid composition of three mixed diets, as determined by column chromatography, with that calculated from tables. The values agreed within 10 per cent on two diets that contained 50 per cent protein from animal sources and 40 per cent from cereals, but values for a vegetarian diet showed many discrepancies. For example, the analytical figures for leucine, lysine, phenylalanine, and arginine were 25 to 50 per cent higher than those calculated.

Numerous microbiological techniques have been proposed in the past for measuring the utilization of intact proteins (112, 113, 114), and two new attempts have been reported. Rodgers *et al.* (115) compared bacteriological

methods with the protein efficiency ratio technique and conclude that the former are unsatisfactory as screening procedures. Conversely, Ford (116) believes that *Streptococcus zymogenes* assay of intact proteins has potential usefulness as a screening procedure.

A new approach to the biological evaluation of protein is that of Longenecker & Hause (117). They tested in dogs the hypothesis that after a test meal the plasma amino acid content should depend not only upon the composition of the dietary protein but also upon the rates of digestion, absorption, and metabolism. At 1, 2, 3, 4, and 5 hr. after the consumption of test meals which contained wheat gluten, casein, or gelatin, 25 ml. of blood were taken from each animal, protein-free filtrates were prepared, and the amino acid composition was determined by column chromatography. The plasma levels for each amino acid, determined after 5 hr., were averaged and the fasting level subtracted to obtain the mean increase. This was then divided by the corresponding figure for the requirement of the dog and multiplied by 100 to give a "plasma amino acid ratio." The values thus obtained ranged from -65 to +58. The most limiting amino acids gave the largest negative or smallest positive numbers, particularly when gelatin and gluten were fed. They corresponded fairly well with chemical estimates of the order of limitation. The values for casein were not as close to the chemical score as were those for gelatin and gluten. Final proof of this technique will be to determine whether it can predict the order in which amino acids must be added to a diet containing a low level of protein in order to stimulate an increase in nitrogen retention or growth. It should be recorded that Frame (118) carried out a similar study in man and failed to find a relationship between the free amino acid composition of plasma and that of a test meal.

PROTEIN-RICH FOODS AND MIXTURES

Recognition of the need for new protein sources has resulted in a substantial increase in studies of this problem, particularly those carried out directly or indirectly under the aegis of the Protein Advisory Group (WHO, FAO, UNICEF) with the co-operation of the Rockefeller Foundation and the National Research Council (119). These studies have been primarily directed toward: (a) the search for and development of new, potentially useful protein sources; and (b) elaboration of protein-rich mixtures from known sources.

Work has continued on the production and testing of edible fish flour. In rat studies, Campbell & Morrison (120) found defatted fish flour to be the equivalent of casein and to be an excellent source of high quality protein with particular promise for supplementation of diets deficient in lysine. Similarly, Metta (121) found that a fish flour had a biological value exceeding that of meat, equal to milk, and less than whole dried defatted egg. In acceptability tests, 26 Indian students could not detect the addition of 3 per cent fish flour to typical Indian dishes.

Lemack *et al.* (122) found that dried ocean perch scales or a pepsin hydrolysate thereof may be of value when used at low supplementary levels to

fish flour. Lea *et al.* (123, 124) report on losses of available lysine in stored herring meal, and Laksessvela (125) points out that heated herring meal may have amino acid deficiencies other than lysine. The effect of nutrient loss in processing of fish flours has previously been emphasized (126, 127). Mann (128) has called attention to a potential supply of meat flour from undersized, underfleshed animals found on overstocked lands in underdeveloped areas. From a practical point of view, it would appear that fish flour has a greater potential than meat flour, since Weiss (129) has estimated the potential yield of fish meat per acre as at least 50 times that of beef. Production of a nutritionally useful product requires control of the process from the selection of the fish to final packaging and distribution of the food—an elementary and obvious fact seemingly ignored in the zealous promotion of certain agencies.

There have been numerous studies of protein-rich preparations or isolates from soybeans and peanuts. Special soybean infant food preparations have been on the market in the United States for many years and have proved to be both nutritious and well-accepted (130, 131, 132). At least three American soybean protein preparations are under scrutiny by the Protein Advisory Group of the National Research Council, as are tofu (a Japanese product), tempeh (a fungus-treated Indonesian soybean product), "Natto" (a Japanese fermented soybean product), and saridele (an Indonesian food product based on soybean and peanut) (133). Gilbert & Gillman (134) found that the addition of either 10 or 20 per cent of soybean to maize protein did not improve growth of the rat as much as did the addition of skimmed milk.

Borchers (135) reports that availability of amino acids is less from raw than from autoclaved soybean meal but that a toxic substance is not necessarily present. Register & Peterson (136) found that rats grow better on starch than on sucrose when the protein is soybean alpha protein, but this was not the case with casein or heated soybean protein.

The potential usefulness of suitably processed peanut flour has been recognized by the Protein Advisory Group of WHO, and the product has been promoted extensively by UNICEF. Solvent-extracted peanut flour for human use is now in commercial production in several countries, including Brazil, England, India, and Nigeria. The results of feeding studies of peanut products vary, largely because of processing differences. Senecal (137) reported that peanut press cake or fish flour, when added to a diet containing millet, results in a mixture of good biological value for the preventive treatment of kwashiorkor. Ladell & Phillips (138) found that six Nigerian adults maintained nitrogen equilibrium on a diet containing per day 33 gm. of peanut flour, 8 gm. of cassava, and 15 gm. of animal protein, and they concluded that peanut flour may be a desirable and acceptable addition to the Nigerian diet. A mixture of 75 per cent peanut flour and 25 per cent skim milk is now being offered commercially for sale in Nigeria (133).

Lal & Roychowdhury (139) studied the biological value and coefficients of digestibility of pressed peanut cake flour in five adult volunteers who received the proteins at a 5 per cent level. The biological value and digestibility of peanut cake flour were 68.7 and 84.2 respectively.

Balasundaram *et al.* (140) reported that unheated and overheated peanut meals are nutritionally inferior to those extracted under mild conditions of heat treatment. Rao *et al.* (141) found that addition of peanut flour improved the growth of rats fed diets based on ragi or jowar, and Tasker *et al.* (142) reported the protein efficiency ratios of various mixtures of peanut flour, sunflower meal, skim milk powder, and bengal gram.

A supplement of a mixture of maize meal and pea flour (2:1) supported growth in a group of children (1.5 to 6 years of age) at a daily intake level of about 2.5 gm/kg body weight, but the performance varied directly with food intake, and the children who did least well were all under three years of age (143). Sesame seed and meals are relatively rich sources of methionine and tryptophan (144), whereas isolated coconut protein has a good lysine content but is low in tryptophan (145). Krishnamurthy *et al.* (146) report a protein efficiency ratio, after eight weeks, for coconut meal of 2.04 compared to 2.29 for dried skim milk.

Joseph *et al.* (147) conclude that dried milk, "multipurpose food," and bengal gram, in that order of efficiency, meet the protein requirements of protein-depleted rats. Regeneration of hemoglobin and plasma protein and repletion of the protein-deficient rat proceeded more slowly on various vegetable protein mixtures than on skim milk (148).

There has been much speculation as to the potential utility of leaf protein as a human food, but few data have been presented. By mechanical means, a protein product can be separated from leaves and pressed into a cake, which had the consistency of cheese. This cake contains 75 per cent protein, 5 to 10 per cent starch, and chlorophyll, and the remainder is mineral matter (149, 150, 151). Guha points out (152) that a similar preparation was used during a famine in Bengal, India, in 1943. Bender (153) questions whether leaf proteins will be a useful supplement to vegetable diets inasmuch as most leaf proteins suffer from a relative deficiency in methionine and cystine. It is obvious that more data will have to be accumulated before this question can be answered. The process at present does not appear to offer immediate promise for wide application.

Studies have been reported on the nutritional improvement of wheat flour or bread by the addition of soya (154); lactalbumin, non-fat dry milk solids, soybean proteins, wheat gluten, and lysine (155); lysine (156); and lysine, threonine, and egg and milk protein (157, 158, 159). In general, the efficacy of a supplement to wheat flour can be predicted on the basis of its lysine content. The rationale for lysine supplementation of wheat foods has been reviewed by Flodin (160), and Moran (161) has critically and exhaustively reviewed the nutritional significance of recent work on wheat flour and bread.

KWASHIORKOR

The syndrome of protein malnutrition (kwashiorkor), which Brock (162) calls "the most serious and widespread nutritional disorder known to medical nutrition science," has been the subject of several symposia during the review period (163 to 166).

In South India, Rao *et al.* (167) estimate at least 120,000 cases of kwashiorkor, and Gopalan (168) emphasized the relationship between this syndrome and avitaminosis-A. Observations on kwashiorkor in New Guinea (169), Sicily (170), the Malay states (171), and Indonesia (172) have been published. Four cases of kwashiorkor have been reported in the United States (173), and Silverblatt & Brown (174) have described a kwashiorkor-like syndrome which was secondary to gastrectomy and associated with "burning feet" in an adult male in the United States.

Senecal (175), Behar *et al.* (176), and Gomez *et al.* (177) have considered various approaches to the treatment and prevention of kwashiorkor in French West Africa, Central America, and Mexico respectively. Frenk (178) reviewed physiological changes occurring in protein malnutrition in childhood and basic points to be considered in its treatment and prevention. Scrimshaw *et al.* (179) drew attention to the relationship between nutrition and infection and conclude that synergism is the dominant interaction that emerges from analysis of the literature.

In efforts to apply nutritional knowledge to the alleviation of this appalling problem, certain promising mixtures of vegetable foodstuffs have been developed and tested in both the prevention and treatment of children with kwashiorkor. The mixture developed co-operatively by the Institute of Nutrition for Central America and Panama (INCAP) and the Instituto Agripecuario Nacional of Guatemala (IAN) is of interest in this respect and is illustrative of the progress in this direction. The considerations basic to planning such mixtures were detailed by Behar *et al.* (176), and the animal studies used in the development of this mixture have been reported (180, 181).

Feeding trials carried out with children recovering from kwashiorkor and in the treatment of children with acute kwashiorkor have been summarized by Scrimshaw & Bressani (182). Currently, INCAP vegetable mixture 9-B, now known as INCAPARINA, contains 29 per cent whole ground maize, 29 per cent whole ground sorghum, 38 per cent cottonseed flour, 3 per cent torula yeast, 1 per cent calcium carbonate, and 4500 I. U. of vitamin A per 100 gm. It has a protein content of some 27 per cent and, according to the FAO reference pattern, a tryptophan score of 61 and a methionine-cystine score of 77. Although, theoretically, lysine should not be limiting, a significant improvement in the growth of rats has been produced by the addition of 0.2 per cent lysine, which suggests that the lysine content of the FAO reference pattern may be too low. INCAPARINA has proved to be highly acceptable to low-income groups in Central America, and the development of this product may serve as a model for the development of similar products in other countries.

Peanuts alone have been found to be relatively unsatisfactory for the treatment of kwashiorkor, but mixtures of peanuts with skim milk (183), with gram and sesame meal (184), with maize flour and skimmed milk (185), and with beans (186, 187) have been useful in the treatment of protein malnutrition. The importance of careful animal testing of proposed mixtures

which contain exotic protein sources is stressed by the report of Fox & Miller (188), who found that the ackee fruit (*Blighia sapida*) contains a riboflavin antimetabolite for the rat.

The enzyme changes occurring during protein malnutrition have been reviewed by Waterlow (189) and by Dean (190). Earlier work on the enzymatic changes in kwashiorkor were detailed by Waterlow & Patrick (191) and by Burch *et al.* (192, 193). In the latter studies, liver biopsy samples and sera of six Guatemalan children with kwashiorkor were analyzed before and after treatment. On treatment, liver protein rose an average of 38 per cent and total lipids fell. Relative to liver protein, xanthine oxidase and D-amino acid oxidase increased appreciably; riboflavin, glycolic acid oxidase, the dehydrogenase of reduced nicotinamide-adenine dinucleotide (NADH), malic dehydrogenase, transaminase, and cholesterol did not. Pyridine nucleotides tended to increase, but not significantly. During treatment, serum protein rose 70 per cent, erythrocyte riboflavin levels doubled, and the serum cholinesterase and amylase activities increased by 100 per cent and 30 per cent respectively. After treatment, all liver constituents except glycolic acid oxidase and xanthine oxidase were about the same as in autopsy specimens from rats or well-nourished American children.

Mukherjee & Sarkar (194) have confirmed the fact that the cholinesterase activity of liver is reduced in the malnourished human; they found, however, that liver catalase and alkaline phosphatase activities decreased during recovery. Stuart *et al.* (195) found that serum cholinesterase levels in marasmic kwashiorkor and marasmus could not be used in differentiating one from the other. DeMaeyer & Vanderborgh (196) conclude that the steatorrhea frequently found in kwashiorkor results from failure to digest, as well as to absorb, dietary fat.

Mexican workers (197) observed low arginine, leucine, and threonine and abnormal phenylalanine-tyrosine ratios in the blood of patients with kwashiorkor. The latter has also been recorded by Holt (198), who suggests that a lack of the enzyme phenylalanine hydroxylase may be involved. Edozien (199) reports that pyruvate is the main keto acid in the blood in kwashiorkor, but in some cases α -ketoglutarate also accumulates. No correlation exists between the severity of the disease and the blood keto acid levels. The aminoaciduria of kwashiorkor (200) has been studied by several other groups (201, 202). Large amounts of β -aminoisobutyric acid are excreted, and in some cases increased taurine and histidine excretions have been recorded. Dried skimmed milk corrected the abnormal pattern, except for β -aminoisobutyric acid. It is considered that the aminoaciduria is largely of renal origin, but that the β -aminoisobutyric acid may be of hepatic origin. Schendel & Hansen (203, 204) suggest that renal function is normal in kwashiorkor but that utilization is affected and the renal threshold for amino acids is exceeded. Edozien *et al.* (205) believe the aminoaciduria to be of renal origin and find, furthermore, that urinary ethanolamine and β -aminoisobutyric acid are increased with a marked reduction of total serum nitrogen. Since the ethanolamine excretion could be reduced by the administration of methionine, it is

suggested that it accumulates as a result of methyl group deficiency. Dean (206) found that a large percentage of the urinary nitrogen of kwashiorkor patients could not be accounted for in terms of the usual nitrogenous constituents and that part of this unknown fraction was composed of abnormal purine-like substances.

Waterlow & Mendes (207) have shown by biopsy before and after treatment, of the pectoral muscle of children with kwashiorkor that the ratio of non-collagen nitrogen to DNA phosphorus increased by 45 per cent, whereas the increase in body weight was only 29 per cent. It is suggested that the discrepancy between muscle cell protein and weight gain may be explained on the basis that gain in tissue protein during recovery is partially masked by loss of water or fat. It was also demonstrated in rats that the sarcoplasmic proteins are reduced in protein deficiency, whereas collagen is relatively increased. An inference is that the loss of protein in protein malnutrition states occurs at the expense of the cellular fraction (208). Waterlow (209) also reports a reduced RNA-DNA ratio in acute malnutrition.

As a result of studies on Jamaican infants and protein-deficient dogs in which radioactive methionine was used, Garrow (210) believes that the protein-depleted organism concentrates protein synthesis in more essential organs. To test more fully this concept, Waterlow (211) measured the incorporation of S^{35} methionine into the viscera, skin, and carcass of normal and protein-depleted rats. He found relatively greater amounts of the isotope incorporated into the liver, brain, and other viscera than into muscle, carcass, and skin. In further studies on Jamaican children (212), the 24 hr. creatinine output doubled during the recovery of malnourished infants. Since this was relatively greater than the increase in body weight, it was concluded that the deficit in muscle mass at the time of admission to the hospital was greater than the deficit in body weight. Since the loss from muscle is relatively much greater than from essential organs such as liver and brain, during recovery a large part of the ingested nitrogen is devoted to the rebuilding of muscle tissue. Vincent & Radermecker (213) found the earliest changes in protein-deficient muscle to be an increase in the number of nuclei in the neurolemma, along with an increase in the size of endothelial nuclei of capillaries where they cross muscle fibers with small collections of lymphocytes around these vessels. In severe protein deficiency, the muscle suffers a loss of striation and an increase in dark nuclei ranged in rows, and, finally, the sarcoplasm disappears.

Numerous studies have been made of the serum proteins in kwashiorkor. El Gholmy *et al.* (214) report hypogammaglobulinemia in Egyptian children with kwashiorkor, which contrasts with the findings of others (215, 216, 217) who report hypergammaglobulinemia. It is suggested that in El Gholmy's group there was a rarity of cirrhotic changes, as judged by liver biopsy, and that associated infections were of short duration. Bassir (218) studied 429 cases of kwashiorkor in Nigeria and concluded that the concentration of gamma-globulin is independent of total serum protein during recovery.

Baptist, DeSilva & Sideek (219) hold that the observation of high gamma-globulin in kwashiorkor is correct, only if it refers to the relative value for this fraction. Gupta & Pohowalla (220) report that the main reduction in a serum protein fraction in kwashiorkor is in the albumin. The results of Gitlin *et al.* (221) suggest that this may be attributable to impaired formation rather than to an increase in its breakdown. Cohen & Schamroth (222) report data which suggest that Africans have a somewhat smaller intravascular pool of albumin with a slower rate of albumin breakdown.

MacDougall (223) described the moderate anemia present in both kwashiorkor and marasmus. The marrow was erythronormoblastic. There was no correlation between degree of anemia and the serum vitamin-B₁₂ levels or serum protein concentrations.

In studies of hyperproteinemia in Panama, Arroyave *et al.* (224) report that all serum protein fractions were higher than normal. They caution against accepting the usual explanation that elevated total serum protein is only attributable to elevated gamma-globulin. Bergot & Bascoulergue (225) feel that the high values for total protein and gamma-globulin found in the sera of many Africans may be related to the incidence of malaria in the population.

Invaluable to those persons interested in protein foodstuffs is the extensive monograph on protein foodstuffs of processed plants, edited by Altschul (226); it details the general properties, chemical and nutritional, of the common protein concentrates of vegetable origin and considers their evaluation. Covering an even wider range of protein foods, including fish, meat proteins, milk, algae, and unicellular organisms, as well as cereal proteins, is the excellent compilation published by the Indian Council of Medical Research entitled *Proteins in Foods* (227). The intense interest of the Indian group in the protein problem is further highlighted by the abstract of papers of the *Symposium on Proteins* (228) in Mysore, which includes technical discussions of the chemistry, biochemistry, technology, and nutritional values of proteins. The subject of the nitrogen requirement has been reviewed in detail by Jacquot & Vigneron (229) as the second in a planned series of four monographs prepared by the French school on amino acids and proteins.

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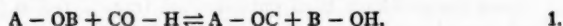
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GLYCOSIDASES AND TRANSGLYCOSIDATION^{1,2,3}

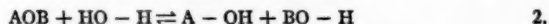
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INTRODUCTION

In recent years, almost all the carbohydrases have been found to be essentially transosylases which catalyse the transfer reaction

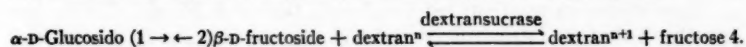
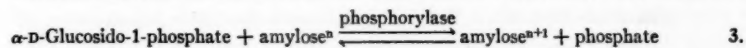


in which A is a glucosyl radical transferred from the donor molecule AOB to an acceptor CO-H, and B is the displaced group and may be a glycone or an aglycone. The reaction of hydrolysis is only a special case in which the acceptor is water:



Transosylation is becoming so classical a notion that the term is employed also for some polysaccharidases, such as α - and β -amylases, for which water is the only acceptor.

Rabate (247) in 1935 was the first to bring clear evidence of a reaction of transosylation; he showed the transfer of "active glucose" from aryl- β -glucosides to aliphatic alcohols by the action of β -glucosidase. But the concept of transosylation is linked to the discovery by Hehre (129) of dextran-sucrase which showed that the synthesis of high polymer homopolysaccharides is possible by enzymatic reactions other than those involving the phosphorylase system. These two systems, however, are not really very different:



In the two cases, one osidic residue linked to the phosphate in glucose-1-phosphate or to fructose in sucrose is transferred to an acceptor molecule to form a polysaccharide.

The discovery by Leloir and co-workers (45, 179, 181) of the synthesis of

¹ The survey of the literature pertaining to this review was concluded in January, 1961.

² The following abbreviations are used: GDP for guanosine diphosphate; UDP for uridine diphosphate; UDPG for uridine diphosphate glucose; F for fructose; G for glucose; Gal for galactose. The glycosidic bond is expressed by an arrow directed from the semiacetalic carbon to the alcoholic group (1 \rightarrow 4); the diosidic bond occurring in sucrose or analogues is expressed by two opposite arrows (1 \leftrightarrow 2).

³ The reviewer wishes to express his appreciation to Dr. R. Austrian for his help in writing the English text and for his valuable suggestions during the preparation of the manuscript.

sucrose from uridine diphosphate D-glucose by an enzyme from wheat germ elucidated another type of transosylation in which the donor molecule is a sugar nucleotide. This reaction has proved to be very important in the synthesis of polysaccharides, chiefly β -polysaccharides and heteropolymers.

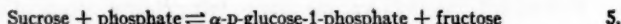
For convenience we shall classify the transosylases arbitrarily into four classes: (a) phosphotransosylases—the normal donor is a glycosyl phosphate (b) true transosylases—the donor is a glycoside and the enzyme functions in the synthesis of polysaccharide; (c) transosylases-glycosidases—the donor is a glycoside but the main action of the enzyme is hydrolytic; and (d) nucleotide-transosylases—the donor is a nucleotide-ose.

Some reviews have been published on transosylation (68, 83, 155, 165). We do not plan to give a general survey of the literature on glycosidases but only to discuss some points in connection with transosylation, the mechanism of action of these enzymes, and the biosynthesis of polysaccharides.

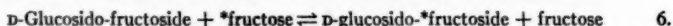
PHOSPHOTRANSOSYLASES

SUCROSE PHOSPHORYLASE

The sucrose phosphorylase (α -D-glucose-1-phosphate \rightarrow sucrose transosylase) from *Pseudomonas saccharophila* catalyses the phosphorolytic split of sucrose (124):



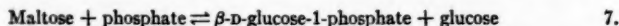
It has been shown that fructose can be replaced by α -L-sorbose, β -D-xyloketose, α -L-araboketose, β -D-rhamnulose (220a), or L-arabinose. In the presence of inorganic ^{32}P -phosphate and glucose-1-phosphate, the enzyme facilitates exchange between labeled and non-labeled phosphate. In the same way, free ^{14}C -fructose is exchanged with fructose from sucrose:



These exchange reactions led Doudoroff, Barker & Hassid (75) to the hypothesis that an intermediary glycosyl enzyme complex is formed and stores the energy of the glycosidic linkage. No such exchange has been demonstrated with muscle phosphorylase (50).

MALTOSE PHOSPHORYLASE

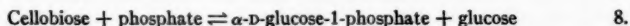
Fitting & Doudoroff (98a) found in *Neisseria meningitidis* an enzyme, maltose phosphorylase (glucose-1-phosphate \rightarrow maltose *trans* α - β -glucosidase), which gives the reaction



There is an inversion of the glycosidic linkage during the reaction, and no exchange occurs. The same enzyme system has been found to function in some strains of lactobacilli (318).

CELLOBIOSE PHOSPHORYLASE

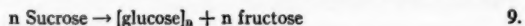
An enzyme has been obtained from three rumen bacteria (13, 142, 267) which catalyses phosphorolytic cleavage of cellobiose. Ayers (13) investigated the cellobiose phosphorylase from *Ruminococcus flavefaciens* and has shown that it also produces an inversion of the linkage:



TRUE TRANSOSYLASES

TRANSGLUCOSYLASES

Dextranucrase.—Dextrans are highly polymerised polysaccharides formed from glucose residues linked in chains in the $\alpha(1\rightarrow6)$ positions with a variable number of branches of various lengths linked in $\alpha(1\rightarrow4)$ or $\alpha(1\rightarrow3)$ positions. Hehre (129) obtained the enzymic synthesis of dextran by an exocellular enzyme preparation from *Leuconostoc mesenteroides* from sucrose:



Similar results have been obtained with preparations from *Betacoccus arabinosaceus* (26) and from *Streptococcus bovis* (18).

Dextrans synthesized enzymatically appear analogous to dextrans obtained from cultures of the organisms from which the enzymes were derived (134). The reaction does not appear to be reversible. Sucrose was long thought to be the unique donor substrate, but Tsuchiya (290) found that maltose, panose, and oligodextrans can also act as glucosyl donors.

Dextranucrase has a high specificity for the transfer to the primary alcohol group at C(6) of free or glycosidically linked glucose. This specificity, however, is not an absolute one. In the presence of a high concentration of sucrose, the enzyme produces oligosaccharides, and one reducing disaccharide leucrose [O-D-glucopyranosyl (1 \rightarrow 5) fructose], which originates in the transfer of glucose on the liberated fructose, has been isolated in crystalline form (270a, 291). Though impure preparations have always been used, strong evidence exists in favour of the production of leucrose by dextranucrase itself (21). Isomaltulose [O- α -glucopyranosyl (1 \rightarrow 6) D-fructose] is formed under the same conditions (266a). Bourne *et al.* (34a) have shown that in the presence of lactose the transfer of glucosyl may occur to the C(2) of the glucose moiety of the lactose molecule producing a branched trisaccharide [-O- β -D-galactopyranosyl (1 \rightarrow 4)-O{- α -D-glucopyranosyl-(1 \rightarrow 2)}-D-glucose].

Dextrans synthesized by an enzyme that acts on sucrose alone have a very high mol. wt. of more than 10^6 (37). Some sugars act as acceptors and thus increase the speed of the reaction and give oligodextrans which terminate in the acceptors. These sugars include isomaltose, maltose, α -methylglucoside, and glucose. Fructose, melibiose, galactose, and leucrose are poor acceptors (162, 291). Bailey *et al.* (20) showed that isomaltosyl- β -fructo-

furanoside is a good acceptor. This finding can be related to the fact that in the presence of sucrose alone, some molecules of sucrose act primarily as acceptor. In very high concentrations of sucrose, the enzyme gives oligodextran containing 0.6 per cent sucrose. This sucrose has been shown enzymatically to be linked at one end of the chain (272). Hehre (133) has obtained the same result with dextran of an average mol. wt. of 60,000 produced by a strain of *Streptococcus*. In presence of such acceptors as α -methylglucoside, one always obtains dextrans of low and high degrees of polymerisation, the ratio of low to high varying in the same direction as the ratio of acceptor to donor, but the distribution is bimodal and not continuous (37, 290).

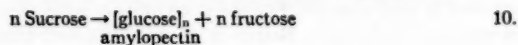
The kinetics of activation by α -methylglucoside and oligodextrans have been studied by Stringer & Tsuchiya (272). In the presence of low concentrations of sucrose, α -methylglucoside acts as a competitive inhibitor, but, when the concentration of sucrose increases, it becomes a powerful activator. It increases the speed of the reaction and the apparent K_m for sucrose (37). The oligodextrans are almost 10 times as active as α -methylglucoside. The K_m for sucrose is given as $1.2 \cdot 10^{-2} M$ at 15° by Stringer & Tsuchiya (272); $3 \cdot 10^{-2} M$ at 30° by Neely (214); and $2 \cdot 10^{-2} M$ at 23° by Hehre (131). The former give a K_A for α -methylglucoside of about $15 \cdot 10^{-2} M$. According to Hehre (134), however, it does not seem that preformed dextran is required to prime dextransucrase.

A study of the pH activity curve of dextransucrase led Neely (214) to implicate the participation of a carboxyl and of an imidazol group in enzyme activity. The results of photo-oxidation experiments favour strongly the participation of the imidazol group in the activity of the enzyme (215).

Following the growth of dextran chains by light scattering, Bovey (37) found evidence to support the single-chain mechanism postulated earlier by Stacey (269) for the synthesis of dextrans with high degrees of polymerisation, but he was led also to postulate a second mechanism which involved multiple chains to explain the broad distribution of the dextrans of lower molecular weight.

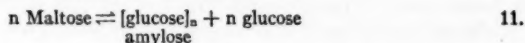
The origin of the branching linkages 1 \rightarrow 3 and 1 \rightarrow 4 is not known. Bailey *et al.* (19) showed that a dextransucrase preparation from *Betacoccus arabinosaceus* contained two activities, one of which accounted for the formation of branches. In cultures the presence of Mg^{++} was necessary for the production of highly branched dextran by this strain (26). With strains of *Streptococcus bovis*, Bailey (18) obtained essentially linear dextran. Pure preparations of enzyme are needed to solve this problem, but Hehre (134) has recently given a very exciting survey of the theoretical possibilities.

Amylosucrase.—This enzyme (sucrose \rightarrow amylopectine trans α -glucosidase), extracted by Hehre (130) from *Neisseria perflava*, catalyses the synthesis of a polysaccharide of the amylopectin type from sucrose:



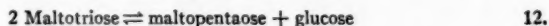
The polysaccharide is very similar to glycogen.

Amylomaltase.—This enzyme (maltose→amylodextrins *trans* α -glucosidase), isolated by Monod & Torriani (211) from a strain of *E. coli*, forms an amylose type of polysaccharide from maltose:



The reaction is reversible. If glucose is allowed to accumulate in the medium, the reaction comes to an equilibrium and dextrans of four to six glucose units are obtained (25). If glucose is continuously oxidised by glucose oxidase, the reaction is shifted to the right and iodine-stainable polysaccharide is obtained (288). Wiesmeyer & Cohn (317) purified the same enzyme from *E. coli* ML 308 and obtained it in the form of a protein homogeneous on electrophoresis and ultracentrifugation. The mol. wt. is 124,000. The enzyme monomer polymerises at low ionic concentration. The pH optimum is 6.9, and the turnover number is 1160 mole maltose/mole enzyme/min at 28° and pH 6.9. They demonstrated conclusively that it is the non-reducing glucose of maltose that forms the polymer.

D-enzyme.—This enzyme was found by Peat *et al.* (236) in potato tubers. It transfers α -glycosyl residues in the maltodextrin series. Maltotriose or longer chains can act as donor substrate but maltose and glucose cannot. Two or more glucosyl residues are transferred at once:



The reaction is reversible. Addition of glucose and iodine-staining chains to D-enzyme produces a decrease of color with iodine and the formation of small dextrans. Glucose can act as an acceptor, but so can methyl- α -glucoside, etc. The transfer is always to C(4). With addition of a glucose-utilising system, the equilibrium is shifted to right so that an iodine-staining amylose-type polysaccharide is produced (298)

Branching factor and Q-enzyme.—These enzymes (amylose→amylopectine *trans* α -glucosidase), the first found in liver and heart by Cori & Cori (55) the second in potato by Haworth *et al.* (127), bring about the transformation of straight-chain amylose to branched amylopectin by the transfer of part of the chain from a (1→4) linkage to a (1→6) linkage. They differ in that the Q-enzyme from potato requires chains of a least 40 residues, transferring a 15 to 20 glucose unit chain, instead of the 6 to 11 unit chain needed for branching factor. Potato Q-enzyme has been crystallised by Gilbert & Patrick (105).

Gunja, Mannors & Khin Maung (119) reported that a similar enzyme from yeast, called yeast-branching enzyme, transforms amylose and amylopectin to a glycogen-type polysaccharide.

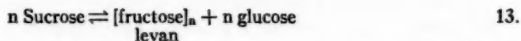
Bacillus macerans amylase.—Discovered by French and his colleagues (101), this enzyme (maltodextrine→cycloamylose- α -transglucosidase) transforms linear chains of amylose into cyclic Schardinger dextrans which contain six, seven, or eight glucose residues known, respectively, as cyclohexa-, cyclohepta-, or cyclooctaamylose. It executes a complex series of transfers

between these cycloamyloses and glucose, maltose, or maltodextrin chains. Glucose can be replaced as an acceptor by some other sugars: cellobiose, sucrose, turanose, glucoheptulose, melezitose, and all sugars in which a glucose is bound only by the glucosidic linkage. All linkages are $\alpha(1 \rightarrow 4)$.

Dextran dextrinase.—This enzyme (dextrin \rightarrow dextran *trans* α -glucosidase), found by Hehre (132) in *Acetobacter capsulatum*, catalyses the transfer of a glucosyl terminal residue from a molecule of dextrin to the terminal position of a molecule of growing dextran and transforms the $\alpha(1 \rightarrow 4)$ linkage to an $\alpha(1 \rightarrow 6)$ linkage.

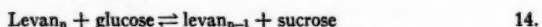
TRANSFRUCTOSYLASES

Levansucrase (sucrose \rightarrow levan *trans* β -fructofuranosidase).—Levans are polysaccharides of very high molecular weight (mol. wt. $\approx 10^6$) made of fructofuranosyl residues linked (2 \rightarrow 6) with branches linked (2 \rightarrow 1). Hestrin and co-workers (9, 136, 137) found enzymes from *Bacillus subtilis* and *Aerobacter levanicum* which synthesize levan from sucrose:

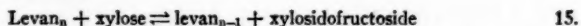


In fact, free fructose is always also formed, as are oligosaccharides (70). The latter include 1 β -fructosylsucrose as the major component, but by transfer to free fructose they also include the two difructoside isomers inulobiose (2 \rightarrow 1) and levanbiose (2 \rightarrow 6), and by transfer to glucose, the three isomers 2-, 3-, and 6- β -fructosylglucose (89).

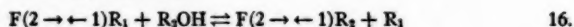
The reversibility of the reaction has been demonstrated. By incubation of the *B. subtilis* enzyme with a mixture of glucose and a levan of mol. wt. of 6000, sucrose is formed (69, 242):



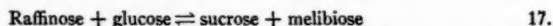
Glucose may be replaced by D-xylose, D-galactose, or L-arabinose, which then leads to the formation of the non-reducing disaccharide analogues of sucrose (237); for instance:



Hestrin *et al.* (139) showed that the formation of sucrose analogues may be obtained by an exchange reaction:

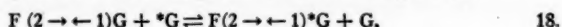


or, as a specific case:



D-Xylose, L-arabinose, D-galactose, melibiose (139), and lactose (10) may replace glucose in this reaction. Proof has been given of the structure of the α -D-xylopyranosyl β -D-fructofuranoside (11) and of the α -D-galactopyranosyl β -D-fructofuranoside formed (90).

Following the speed of the exchange reaction with ^{14}C -glucose,



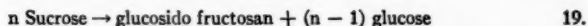
Péaud-Lenoël (239) showed that it works very fast, much faster than the reverse reaction of levan synthesis (Reaction 14). The exchange reaction is feebly inhibited by levan.

These results indicate, as shown by Péaud-Lenoël (237), that, to give an active donor substrate, the sugar linked by a diosidic linkage to the fructofuranosyl rest must have the OH group of C(2) in the *cis* position with the α position of the semiacetal linkage and the OH group on C(3) in the *trans* position. In an extensive study, Hestrin & Avigad (138) found that aldoses in which the hydroxyl positions on C(2) and C(3) are reversed may react feebly.

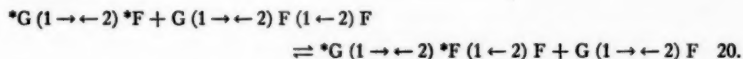
Small levans act as primers and accelerate the rate of the synthesis reaction and increase the ratio of levan formed to free fructose (238). Levan of mol. wt. of 6000 is the most effective. With highly purified enzyme, the K_m with sucrose is 5.10^{-2} M and the K_A for the primer levan is 0.33 to $0.6 \cdot 10^{-2}$ M. At very low concentrations of sucrose, however, high concentrations of levan added as primer become inhibitory (69).

There is no information about active groups or about branching. A homogeneous protein enzyme from *B. subtilis* appears to give branched levans and has a definite levanase effect. The same enzyme brings about hydrolysis of glucosidosorbofuranoside and of glucosidoxylketoside. The K_m for the former is about 1.10^{-1} M (69).

Transfructosylases from Jerusalem artichoke.—Inulosucrase (sucrose→inulin *trans* β -fructofuranosylase) was found by Dedonder (67) in an extract of the tubers of a Jerusalem artichoke strain.



The trisaccharide, 1^F-fructosylsucrose, is formed in greater amount than the other oligosaccharides of the series. Edelman & Bacon (85) found in Jerusalem artichoke an enzyme which does not act on sucrose alone but catalyses the transfer of fructofuranosyl residues from inulin to sucrose to give small fructosans. Recently, Edelman (84) also found in these tubers an enzyme which transfers fructosyl from the trisaccharide, 1^F-fructosylsucrose, to sucrose:



The reaction is very much faster than any other transfructosylation so far discovered in tuber extracts.

TRANSOSYLASES-GLYCOSIDASES

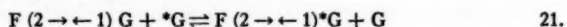
TRANSFRUCTOSYLASES

Following the course of the hydrolysis of sucrose by yeast invertase by paper chromatography, Bacon & Edelman (15) and Blanchard & Albon (33) demonstrated the formation of some di- and trisaccharides which are subsequently degraded by the enzyme to give glucose and fructose as the only terminal products. Transfer of fructosyl to sucrose gives 6^F-, 1^F-, and 6^G-

fructosylsucrose; transfer to glucose gives O- β -D-fructofuranosyl-(2 \rightarrow 6) glucose, and transfer to fructose yields levanbiose and inulobiose. Sucrose may be replaced as a fructosyl donor by raffinose or by O-methyl- β -fructofuranoside. Sugars with a primary alcohol group, such as galactose, mannose, mannitol, or primary alcohols (methyl, ethyl, etc.) can function as acceptors (83). Hydrolysis and transfer are carried out by the same enzyme. One highly purified preparation electrophoretically homogeneous obtained by Fischer *et al.* (94, 95) shows both types of activity.

Mold invertases acting on sucrose also produce oligosaccharides, but the pattern is somewhat different. In the first part of the reaction little fructose is liberated, the fructosyl residues being transferred preferentially to acceptors other than water with 1 $^{\circ}$ -fructosylsucrose as the main product (28). Pazur (224, 225) obtained the same results with *Aspergillus oryzae* enzyme and described it as a specific transfructosidase; but in the end this enzyme gives free glucose and fructose. Bealing (27) purified an extract from *Penicillium spinulosum* and obtained similar results.

Yeast and mold invertases differ in that the latter transfer preferentially to the C(1) of the fructosyl part of sucrose. Yeast invertases are inhibited by glucose; mold invertases are not. Edelman (82) showed that mold invertase in the presence of sucrose and ^{14}C -glucose catalysed a rapid incorporation of radioactivity in the glucose moiety of sucrose:



The enzyme catalyses also the formation of sucrose from raffinose and glucose (38). These reactions are exchange reactions of the same type as the one obtained with sucrose-phosphorylase or levansucrase and may fit the glycosyl-enzyme theory. With this enzyme, however, it was impossible to obtain sucrose from O-methyl β -D-fructofuranoside and ^{14}C -glucose. Yeast invertase does not catalyse an exchange reaction and, with ^{14}C -glucose and sucrose, incorporates the radioactivity into the reducing disaccharide, O- β -D-fructofuranosyl (2 \rightarrow 6) glucose.

Invertases of higher plants which are β -fructofuranosylases have been shown to have transferring action and to give 1 $^{\circ}$ - and 6 $^{\circ}$ -fructosyl sucrose from sucrose (5, 246).

TRANS- α -GLUCOSYLASES

Glucosido-invertase.—Honey invertase is a glucosido-invertase. Acting on sucrose, it gives six oligosaccharides, among them maltose and α -maltosyl β -D-fructofuranoside (4 $^{\circ}$ -glucosyl sucrose). Acting on maltose, the enzyme produces a series of oligosaccharides, as does amylomaltase (315, 316).

Invertase from hog intestine purified by ion-exchange column chromatography (64) acts on sucrose and maltose. In sucrose solution, at least four oligosaccharides are formed by transfer of glucose and one of them is 4 $^{\circ}$ -glucosylsucrose (66).

Maltases.—Transferase activity of maltase has been demonstrated by

Pan *et al.* (221 to 223) with an extract of *Aspergillus niger* acting on maltose. They isolated a trisaccharide, panose, which was shown to be O- α -D-glucopyranosyl (1 \rightarrow 6) O- α -D-glucopyranosyl (1 \rightarrow 4) D-glucopyranose. Pazur & French (229) with a maltase from *A. oryzae* obtained one disaccharide, isomaltose; two trisaccharides, panose and dextrantriose; and one tetrasaccharide, α -dextrantriosyl-D-glucose. It appears that the general pattern of transfer by maltases from molds is transfer from (1 \rightarrow 4) linkages to (1 \rightarrow 6) linkages, although *A. oryzae* enzyme can produce nigerose α (1 \rightarrow 3) (228). Crystalline *A. oryzae* maltase has been obtained by Sugawara *et al.* (279), and its specificity has been studied by Matsuhima (200). The same pattern of specificity has been found with an enzyme prepared from liver extract by Lumkomskaia (190) which leads to the formation of isomaltose, panose, nigerose, and a series of other oligosaccharides. Abdullah & Whelan (1) named T-enzyme, a potato enzyme, which produces panose from maltose. The enzyme has been fully separated from D-enzyme.

Maltases were obtained from bovine blood by Miller & Copeland (205) and from hog liver by Giri *et al.* (107). These maltases transfer to C(4), as does amyloamylase from *E. coli*, giving maltotriose and maltotetraose. Stetten (271) investigated the liver enzyme and showed that it transferred glucosyl from maltose, maltotriose, or glycogen to glucose, maltose, or higher dextrans. Oxidising glucose by glucose oxidase led to the production of higher oligosaccharides. A similar enzyme has been found by Nigam & Giri (216) in the higher plant *Phaseolus radiatus*.

Manners (196) found an intermediate type of transferring maltase in marine algae, brewers' yeast, and the protozoan *Tetrahymena pyriformis* which can use hydroxyl groups at both C(4) and C(6) as acceptors.

An enzyme of rat liver catalyses transglucosylation from maltose to riboflavine and to certain other isoalloxazines (314). Enzymes from *A. oryzae* and *E. coli* (157) may also produce riboflavinyol maltosaccharides by further transfer to riboflavinyol glucoside [*E. coli* extracts also catalyse the transfer of β -fructofuranosyl residues from sucrose to riboflavin (158)].

Other α (1 \rightarrow 4)-glucosylases.—It does not appear that α -amylase, β -amylase, or γ -amylase (amyloglucosidase) can transfer glucosyl or polyglucosyl residues to acceptors other than water. There is a report by Sawai (261) of an amylase of *Candida tropicalis* var. *japonica*, which seems to be a γ -amylase, that hydrolyses maltose, hetero- α -glucosides, and soluble starch, and transfers the glucosyl moiety of α -phenyl glucoside to glycerol. These activities could not be separated by acidic, basic, or thermal treatment, by zone electrophoresis, or differentiated by inhibitors.

Trehalase (α, α' 1-1 glucosidase) has been isolated and purified from *Galleria mellonella* (156). Trehalases from mycobacteria and from *E. coli* K 12 are inhibited by trehalosamine (104). Enzyme from *Phormia regina* has been purified and shown to be inhibited by trihydroxyethanolamine (102). Hog intestinal trehalase, purified on a diethylaminoethyl cellulose column, lacks transglucosylase activity (65).

$\alpha(1\rightarrow6)$ -Glucosylases.—Muscle amylo 1-6 glucosidase (58) and R-enzyme (235) are debranching enzymes that act on glycogen or amylopectin, the former as an exoglucosidase and the latter as an endoenzyme. They do not seem to transfer to acceptors other than water. The same is true of oligo 1-6 glucosidases or limitdextrinases. None of these enzymes hydrolyses dextrans. Dextranases have been reviewed by Fischer & Stein (96). Dextranases from molds are endodextranases that act by random cleavage of the dextran molecule. According to Kobayashi (161), the products are liberated with inversion in the β configuration. Dextranases from mammalian cells are exodextranases that degrade dextran by endwise splitting of glucose residues up to branching points, leaving a residual dextran (255). The enzyme has been used to characterize dextrans from different origins (257). As shown by Sery & Hehre (266), a preparation from *Bacteroides* sp. displays endo- and exodextranase activities which can be separated at appropriate pH. Bailey & Clarke (22) obtained an enzyme from *Lactobacillus bifidus* which acts only as an endodextranase, splitting glucose chains at least seven glucose units long. It produces only isomaltose, isomaltotriose, and so on, from *Streptococcus bovis* linear dextrans; but, acting on branched *Leuconostoc* dextrans, it gives branched oligosaccharides, thereby providing a tool for the structural analysis of these dextrans (23).

TRANS- β -GLUCOSYLASES

These enzymes are isolated chiefly from microorganisms. Cellulolytic molds frequently secrete more than one β -glucosidase, depending upon the strain and conditions of growth; i.e., cellulase, cellobiase, and aryl- β -glucosidase. For instance, *Stachybotrys atra*, on which Youatt & Jermyn (325, 326) have worked for many years, first gave a cellulase which hydrolysed carboxymethyl cellulose. Then the strain was modified, and, if grown on a medium that contained cellulose, produced cellobiase which acted on cellobiose and cellodextrins. If grown on other substrates, it gave an aryl- β -glucosidase that had no effect on cellobiose.

Crook & Stone (63) have shown that purified cellobiase from *A. niger*, a crude filtrate from *Myrothecium verrucaria*, and a preparation from *Helix pomatia* acting on cellobiose give a series of oligosaccharides of the cellodextrin type. Prolongated incubation leads to the formation of glucose as the sole final product. Reviewing the results obtained with enzymes from higher plants, marine algae, and molds, Manners (196), indicated that the following oligosaccharides are formed: gentiobiose, laminaribiose, sophorose, gentiotriose, cellotriose, 6- β -glucosylcellobiose, and 3- β -glucosylcellobiose. Transfer of a β -glucosyl residue to hydroxyl groups at C(6), C(3), or C(2) of glucose or to C(4), C(6), or C(3) of the non-reducing residue of cellobiose is indicated. Cellobiase of *Cladophora rupestris* transfers to xylose and gives 3-O- β -D-glucopyranosyl-D-xylose (77). The specificities of cellobiases are rather broad. Duerksen & Halvorson (76) purified an enzyme of *Saccharo-*

myces cerevisiae which acts on arylglucosidases and cellobiose but which has no transferring activity (see also 144).

The properties of cellulases, even those of the same origin, appear to be very controversial (312). Whitaker and co-workers (287, 313) nevertheless obtained a highly purified preparation (mol. wt. = 63,000). Cellotriose and cellotetraose, obtained when this enzyme reacts with large cellobextrins and ^{14}C -cellobiose, display low specific radioactivity, showing that transfer occurs in their formation (311).

The transfer of β -glucosyl residues by aryl- β -glucosidases was the first transosylation process brought to light by Rabate in 1935 (247). He showed that glucosyls coming from phenolic glucosides reacted with primary alcohol to form the corresponding glucosides. Transferring activity of β -glucosidase from almond emulsin have been investigated by Courtois & Leclerc (61). Properties of the aryl- β -glucosidases from *Myrothecium verrucaria* (123) and *Stachybotrys atra* (151) have been studied extensively. With *p*-nitrophenyl glucoside as a donor, primary alcohols (but not glycerol) will act as acceptors in the *M. verrucaria* system. This latter has a marked preference for alcohol rather than water as an acceptor substrate. Retention of configuration has been verified by isolation of methyl- β -glucoside. The β -glucosidase from *S. atra* transfers β -glucosyl to glycerol.

Nisizawa & Hashimoto (217) found in the fungus *Irpex lacetus* an enzyme which, when acting on *p*-nitrophenyl β -cellobioside effects the transfer of cellobiosyl to methanol.

TRANS- β -GALACTOSYLASES

Aronson (8) and Pazur (226) showed that β -galactosidases of *Saccharomyces fragilis* and *E. coli* acting on lactose synthesize some oligosaccharides. Wallenfels (304) isolated, during hydrolysis of lactose by an *Aspergillus flavus* β -galactosidase, a trisaccharide, galactosidolactose, two disaccharides, a galactosido-glucose, and a galactosido-galactose. Pazur (227), using the action of the *S. fragilis* enzyme on lactose, obtained and characterized four oligosaccharides: Gal (1 \rightarrow 6) G; Gal (1 \rightarrow 6) Gal; Gal (1 \rightarrow 6) Gal (1 \rightarrow 4) G; and Gal (1 \rightarrow 6) Gal (1 \rightarrow 6) G. The transfer is chiefly to the hydroxyl at the C(6) position of the acceptor. Sucrose is a good acceptor and gives the β isomer of planteose (6 β - β -galactosyl sucrose) (230 to 232).

As a result of the brilliant researches of Monod's group (208, 209) on protein synthesis concerned with *E. coli* β -galactosidase, this enzyme has been studied most extensively. Cohn & Monod have shown that the enzyme preparations from *E. coli* (ML or K₁₂), *Aerobacter aerogenes*, and *Shigella sonnei* are not distinguishable by their specificity and immunological behaviour.

E. coli β -galactosidase was highly purified by Cohn (51), who found a mol. wt. of 700,000, a turnover number of 4000 moles *o*-nitrophenylgalactoside/sec/mole enzyme and, from equilibrium dialyses, five active sites per

mole. Wallenfels *et al.* (307) obtained the crystallized β -galactosidase from the strain ML 308, and Hu *et al.* (143) from the strain ML 309. Wallenfels has reviewed the general properties of the enzyme (306) and its transferring properties (301). He found that, in the galactosyl transfer reaction, the enzymes of *E. coli* and snail behaved similarly, but those from calf intestine and *A. flavus* were different (305).

The molecular weight of the crystalline enzyme from *E. coli* is near 750,000. Six amino end groups (four threonine and two glutamic acid) were found for this molecular weight (302). There is no indication of a prosthetic group. Na^+ and K^+ are activators; the former increases the speed of hydrolysis of *o*-nitrophenyl galactoside but reduces that of lactose, whereas the latter increases the speed of hydrolysis of lactose. Mg^{++} is needed for the fixation of *o*-nitrophenyl galactoside on the enzyme (251).

The enzyme is a typical SH enzyme. Quantitative inhibition with *p*-mercuribenzoate shows that the number of SH-reacting groups essential for activity varies with temperature: two at 5°, four at 20°, and ten at 40°. Using the ultracentrifugal technique and thionitrophenyl galactoside, a competitive inhibitor, Wallenfels (301) found 1.9 binding sites at 5° and 4.7 at 22° per 100,000 mol. wt. units. He concluded from these results that every active site needs an SH group. There is a big discrepancy with Cohn's results. From the two pK values 5.8 to 6.2 and 7.8 to 8.6, Wallenfels thinks that the active groups are an imidazole ring and SH.

The crude and crystallized enzymes give essentially similar patterns in transfer reactions (307). Of the various isomeric galactosido-glucoses, the isomer which is produced in greatest yield is the most rapidly hydrolyzed. With the enzyme from *E. coli*, the first isomer to appear is the (1→6), and then the (1→4) and the (1→3) can be obtained. With a preparation of the enzyme from calf intestine purified 2100 times and containing 60 per cent carbohydrates, this order of hydrolysis is reversed (301).

As shown by Wallenfels, the glycone specificity is rather narrow. The hydroxyls at C(2), C(3), and C(4) must be free, and in the galactose position. The OH group in position 6 may be replaced by H (β -D-fucoside) and the CH_2OH by H as in α -L-arabinosides (210, 301). With nitrophenyl α -L-arabinoside as donor and glucose as acceptor, three arabinosidoglucoses are formed: (1→6) (vicianose), (1→4), and (1→3). The (1→3) isomer is formed in higher amount at the higher rate but disappears soon after (303). A great number of sugars may be acceptors; sucrose is a good one and gives mainly 6^G- β -galactosyl sucrose (301).

β -Galactosidase has been reported to be present in every organ of the mouse and in L-cells of murine origin in tissue culture (195).

TRANS- α -GALACTOSYLASES

The transferring action of α -galactoside has been reviewed by Courtois (59). Blanchard & Albon (33) with a yeast enzyme acting on melibiose obtained mannanotriose (99). An enzyme from coffee seeds makes α -methyl-D-

galactopyranoside from melibiose and methanol (6). Courtois *et al.* (60, 62), using α -phenylgalactoside as donor has shown that the transfer occurs selectively to a primary alcohol group; with mannose as an acceptor, epimelibiose [α -galactopyranosyl (1 \rightarrow 6) D-mannose] is formed by enzymes from coffee seeds or *Penicillium paxillus*. With sucrose as an acceptor and enzymes from coffee or lucern, raffinose is obtained (7, 62).

TRANS- β -GLUCURONOSYLASES

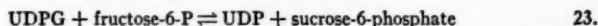
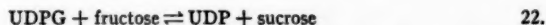
Mammalian glucuronidase may transfer β -glucuronosyl residues from aryl and alicyclic glucuronides to aliphatic alcohols and glycols. High concentration (2 or 3 M) of acceptor is required (98).

NUCLEOTIDES-TRANSOSYLASES

The discovery by Leloir *et al.* (176) of a sugar nucleotide, uridine diphosphoglucose, in yeast in the course of their study of galactose utilization opened a new field in carbohydrate biochemistry. A great number of interconversions between sugars occur only when they are linked to a nucleotide phosphate. The interconversion between UDP-D-glucose and UDP-D-galactose has been shown by Leloir (175a) and Trucco (289a) to occur by the reaction of an enzyme from yeast extract, UDPG-4-epimerase. Strominger *et al.* (275) found in liver extract an enzyme which oxidises UDP-D-glucose in UDP-D-glucuronic acid in the presence of NAD.⁴ Hassid, Neufeld & Feingold (125) have reviewed the main pathways of interconversion in higher plants: UDPG to UDP-D-galactose, UDP-D-glucuronic acid, UDP-D-galacturonic acid, UDP-D-xylose, and UDP-L-arabinose. Strominger (274) in an extensive review of the functions of nucleotides in metabolic reactions, has summarised the known facts of sugar nucleotide occurrence and biosynthesis. In addition, sugar nucleotides function as glycosyl donors in many transglycosylation reactions (240) which lead to the synthesis of glycosides, oligosaccharides, and polysaccharides.

OLIGOSACCHARIDES

Leloir and co-workers (45, 179, 181) obtained with an enzyme from wheat germ sucrose and sucrosephosphate from UDPG and fructose or fructose-6-phosphate:



Two different enzymes are involved. The enzyme catalysing the synthesis of free sucrose was found in many materials of plant origin. Bean & Hassid (29)

⁴ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH₂), for triphosphopyridine nucleotide, reduced form (TPNH).

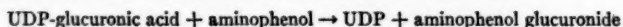
found the same enzymic activities in soluble preparation of green peas and showed that these preparations produce two other disaccharides from UDPG and D-xylulose or from UDPG and D-rhamnulose: D-glucosyl-D-xylulose and D-glucosyl-D-rhamnulose.

An enzyme from yeast leads to the formation of trehalose-phosphate from UDPG and glucose-6-phosphate (44, 178).

Schambye *et al.* (262) have shown by *in vivo* experiments that in the lactose synthesis the glucose part arises from blood glucose but that the galactose comes from the hexose-phosphates produced in the mammary glands. Gander, Peterson & Boyer (103) bring strong evidence that homogenate of mammary glands synthesized lactose phosphate from UDP-galactose and glucose-1-phosphate.

GLUCOSIDES

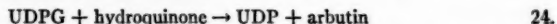
Dutton & Storey (81) showed that the synthesis of glucuronides in liver extract took place in the presence of UDP-glucuronic acid



The enzymatic activity is associated with particulate components of the cytoplasm. Such microsomal preparations may transfer glucuronosyl residues to steroids, thyroxine, and phenolphthalein (149). UDP-glucuronic acid acts as a glucuronic acid donor to carboxylic acids with the resultant formation of ester glucuronides (78). This liver system synthesizes glucuronides with a numerous series of acceptors, including the synthesis of N-glucuronides (12, 79, 118, 264). Dutton & Stevenson (80) found similar particulate enzymes in kidney cortex and gastric mucosa of guinea pig and mouse.

A similar system has been found by Marsh in plants (French bean leaves) in which galacturonic acid is transferred from UDP-galacturonic acid to quercetin (199). Extracts of leucils catalyse the synthesis from UDP-glucose of the anthranilic acid glucoside (150, 282, 283).

Yamaha & Cardini (320) found in wheat germ two soluble enzymes which they separated. One, called UDPG-diphenol transglucosidase, transfers glucose to diphenols, giving β -glucosides:



The rate of reaction decreases with varying acceptors in the order: hydroquinone > hydroxyhydroquinone > methoxyhydroquinone > resorcinol > pyrogallol > pyrocatechol. The other enzyme transfers one more glucose from UDP-glucose to β -phenylglucosides. There are no other acceptors. Chains of more than two rests are not formed (321).

Incorporation of ^{14}C -galactose from UDP- ^{14}C -galactose in the lipid fraction has been shown to occur with a particulate preparation from rat brain (42). Cleland & Kennedy (47) found a system in brain which synthesizes galactosyl sphingosine from UDP-galactose.

POLYSACCHARIDES

Chitin.—Glaser & Brown (110) found chitin synthetase activity in extracts from *Neurospora crassa*. With UDP-acetylglucosamine labeled with ^{14}C in the acetylglucosamine moiety and with soluble chitodextrins, the extract produced an insoluble high polymer which contained acetylglucosamine and which, on hydrolysis by chitinase, gave chitobiose and acetylglucosamine. Incorporation of radioactivity reaches 22 per cent of that contained in the added UDP-acetylglucosamine. The enzymatic activity is found in granules which sediment from 2000 to 140,000 g. By treatment of the particulate preparation with *n*-butanol, a soluble extract with low activity is obtained; but, with this preparation, reversibility of the reaction has been shown. Recent report of the occurrence of UDP-acetylglucosamine in crustacean tissues enhances the probability that the same enzymatic process is involved in the synthesis of crustacean chitin (191).

Cellulose.—Glaser (108), using a particulate extract of *Acetobacter xylinum*, incorporated labeled glucose from UDP-glucose into cellulose in the presence of celloextrins. The amount of incorporation is very low. Purification of the radioactive cellulose and hydrolysis by cellulase leading to labeled cellobiose provide proof, however, of the synthesis of cellulose. Attempts to solubilize enzyme with digitonine give a very labile preparation. Klungsöyr (160) obtained an extract of *Acetobacter xylinum* which synthesizes a cellulose-like material from UDP plus celloextrins. These results would imply a reversible mechanism of transosylation.

Péaud-Lenoël (241) has shown by *in vivo* experiments that radioactivity incorporated into cellulose of growing wheat roots is displaced by decreasing the soluble carbohydrate pool or by isotopic dilution of this pool with non-labeled sucrose. These results suggest also a reversible mechanism of transosylation.

Brummond (41) obtained the formation of an insoluble material with a soluble enzyme from spinach acting on UDP-glucose. UDP was formed. The precipitate was insoluble in water, 1M HCl, and 1N NaOH. The author suggests that the precipitate is an insoluble carbohydrate polymer.

β (1-3)-Glucan.—In an unsuccessful attempt to obtain synthesis of cellulose by mung bean (*Phaseolus aureus*) extract, Feingold, Neufeld & Hassid (91) synthesized another polysaccharide which is widely distributed in plants, β (1-3)-glucan (laminaran from seaweeds, glucan from yeast cell walls, and callose from higher plants). The structure of the polymer has been established. By extraction with digitonin, a soluble enzyme preparation has been obtained from mung bean particles which synthesizes the polysaccharide from labeled UDP-glucose with liberation of UDP. The yield varies from 20 to 85 per cent. The reaction requires Mg^{++} and carbohydrate activators (D-glucose, cellobiose, maltose, sucrose, laminaridextrins) which do not appear to be incorporated in the product.

Pentosans.—An enzyme preparation from asparagus shoots catalyses the

transfer of xylosyl units from UDP-xylose to $\beta(1\rightarrow4)$ -linked oligosaccharides with degrees of polymerisation from 2 to 5. Only one xylosyl unit is transferred to each oligosaccharide; this results in oligosaccharides with degrees of polymerisation ranging from 3 to 6. This result seems to be attributable to the very low affinity of the transferring enzyme for the xylo-oligosaccharides. On incubating UDP-pentose with asparagus particles, one obtains a xyloside insoluble in water but soluble in hot 80 per cent alcohol. The acceptor is probably bound to particles (92).

Glycogen.—Leloir & Cardini (180) have shown that a particulate preparation from liver catalyses the synthesis of glycogen from UDPG. The same activity has been found by other workers in muscle (126, 252, 296) and in brain (39, 40). Leloir *et al.* have examined the properties of the muscle (183) and liver system (182). The muscle enzyme is extracted by homogenisation and purified by acidic precipitation. It requires a primer: soluble glycogen is the best one. The enzyme system is activated by glucose-6-phosphate and also by galactose-6-phosphate and glucosamine-6-phosphate, but these activators are not at all incorporated. When the ^{14}C -glycogen produced from UDP ^{14}C -glucose is treated with β -amylase, all the radioactivity is found in maltose. This result supports the $\alpha(1\rightarrow4)$ nature of the linkage which has been formed. Hauk & Brown (126) found similar results using phosphorylase.

On centrifugation, the liver enzyme sediments with the small particle fraction. This fraction may be divided in two parts: microsomes and particulate glycogen. The enzymatic activity is found in the particulate glycogen fraction. Washing of this fraction with soluble starch eliminates inactive proteins and brings about a 300-fold purification. The optimum pH for enzyme activity is 8.4. The K_m for UDPG is $4.8 \cdot 10^{-4}$ M. As with the muscle system, glucose-6-phosphate, galactose-6-phosphate, and glucosamine-6-phosphate are activators of the incorporation of ^{14}C -glucose from UDP- ^{14}C -glucose into glycogen. Glucose-6-phosphate also protects the enzyme against inactivation during preincubation. These substances are not incorporated. It seems possible to obtain partial solubilization of the enzyme by washing the particulate fraction with soluble glycogen (KOH-glycogen).

Since it has been shown that increasing phosphorylase activity leads to glycogen breakdown (56, 249, 250), it is most probable that the UDPG-glycogen transglycosylase is the physiological glycogen-synthesising system (see also 171). Studies of pyridoxine deficiency (145), of the action of insulin on UDPG glycogen transglycosylase (297), and especially of a glycogen disease shown to be attributable to absence of phosphorylase in muscle (207) support this view.

Olavarria (218) has shown that the oligosaccharides which always occur during the synthesis of glycogen from UDPG by the "particulate glycogen" enzyme proceed from a degradation of the synthesised glycogen.

Hyaluronic acid.—Glaser & Brown (109) produced the first evidence of the incorporation of labeled glucuronic acid and of N-acetylglucosamine, derived from UDP-glucuronic acid and UDP-N-acetylglucosamine, re-

spectively, into hyaluronic acid by a particulate cell-free extract of Rous sarcoma. An important part of the incorporated radioactivity was dialysable and found in oligohyaluronates. Markowitz, Cifonelli & Dorfman (197, 198) were able to confirm these results by using a cell-free extract of a group A *Streptococcus*. They obtained with tritiated substrate the incorporation of the radioactivity of N-acetylglucosamine from UDP-N-acetylglucosamine into the acetylglucosamine part of hyaluronic acid in presence of non-labeled UDP-glucuronic acid and the incorporation of the radioactivity of glucuronic acid from UDP-glucuronic acid into the glucuronic part of hyaluronic acid in presence of non-labeled UDP-N-acetylglucosamine. The radioactivity incorporated into hyaluronic acid was not modified by repurification. The yield reached 12.5 per cent for the transfer of glucuronic acid. Mg^{++} was needed. The authors suggest several possibilities for the mechanism of alternate incorporation of the two monomers.

Type III pneumococcal polysaccharide.—Smith *et al.* (268) found that a cell-free extract of *Diplococcus pneumoniae* Type III synthesises polysaccharide from UDP- ^{14}C -glucose and UDP- ^{14}C -glucuronic acid. Proofs are given of the identity of the polysaccharide formed with natural Type III polysaccharide (a cellobiuronic polymer) by its reaction with specific anti-serum and its hydrolysis by a specific depolymerizing enzyme. Using non-capsulated strains of Type III and transformation by DNA of capsulated strains of Type I or XXXIII, Mills *et al.* (206, 206a) have shown that blocks in the transformation from UDPG to UDP-glucuronic acid are responsible for the absence of production of the polysaccharide.

Another interesting transosylation has been shown by Kornberg *et al.* (163). In *E. coli* infected by the bacteriophage T_2 , an enzyme is formed which transfers glucose from UDPG directly on the hydroxymethyl cytosine in the DNA.

To date, glycosyl transfers from sugar nucleotides are only known with uridine nucleotides. It is tempting, however, to suggest that guanosine diphosphate sugars and thymidine diphosphate sugars may have similar functions. GDP-mannose has been isolated from yeast by Cabib & Leloir (43) and from hen oviduct by Strominger (273). It has been recently found in a red alga together with GDP-L-galactose (277). Ginsburg (106) has shown the formation of GDP-L-fucose from GDP-D-mannose by an enzymic preparation of *Aerobacter aerogenes* in presence of $NADPH_2$ (106). GDP-colitose has been obtained by Heath (128) from *E. coli*, and Pontis, James & Baddiley (245) found GDP-glucose and GDP-fructose in *Eremothecium ashbyii*.

Strominger & Scott (276) isolated from a diaminopimelic acid-requiring mutant of *E. coli* three thymidine diphosphate sugars. The sugars were not identified but appeared to be deoxyhexoses. At the same time, Okazaki *et al.* (219, 220) found thymidine diphosphate-rhamnose and another thymidine diphosphosugar in *E. coli*. Baddiley & Blumson (14) obtained thymidine diphosphate-mannose and -rhamnose from the mycelium of *Streptomyces griseus*. Pazur & Shuey (233) studied the role of thymidine

nucleotides in the synthesis of L-rhamnose by an extract of *Streptococcus faecalis*, and Kornfeld & Glaser (164) obtained the enzymic synthesis of thymidine diphosphate-glucose and -rhamnose by an extract from *Pseudomonas aeruginosa*, an organism which synthesises a rhamnolipid in the medium. In this case, the participation of thymidine diphosphate-rhamnose in the transfer of rhamnosyl residue is pretty clear. It is also most probable that GDP-mannose may play a part in the synthesis of yeast mannan or mucopolysaccharides of the hen oviduct and that GDP-colitose is implicated in the formation of the O antigen of *E. coli*.

GENERAL PROPERTIES OF TRANSOSYLATION ENZYMES

It is important to note first that many of these enzymes have not been purified and that some controversies about specificity will probably be resolved after further work has been done.

Other than the phosphorylases, the α - and β -amylases, and the Q-enzyme, very few transosylation enzymes have been crystallised. Rapid progress is now being made. *E. coli* β -galactosidase (143, 307) and neuraminidase (2, 265) have been crystallised. As for lysozyme, which has been known in crystalline form for many years, its specific activity has been demonstrated to be that of a glucosaminidase (31, 259).

In pure but amorphous form, electrophoretically homogeneous, we have now amylomaltase from *E. coli*, (317) and yeast α -glucosidase (121, 244).

In some cases, it seems very difficult to separate transosylases from associated carbohydrate. Such is the case for yeast invertase, purified by Fischer and co-workers (94, 95). The enzyme is contaminated with mannan; separation may be obtained by adsorption of the enzyme on bentonite; but the enzyme cannot be eluted from the bentonite in active form. β -Galactosidase from calf intestine mucus purified by Wallenfels (301) contains 60 per cent carbohydrate. In some cases, it seems that the carbohydrates are structurally linked to the enzyme. In Taka-amylase, a chain containing eight mannoses and one xylose is linked by the xylose to a series of the amino acid chain of the enzyme (293).

There is no evidence that these enzymes require prosthetic groups, except in phosphorylases. The evidence based on amino acid balance, however, cannot give absolute proof of their absence and we must remember that almost 20 years separate the discovery of muscle phosphorylase by Cori and co-workers (53, 54) and the finding of pyridoxal phosphate as an active part of the enzyme molecule (24, 57). Yunis, Fischer & Krebs (327), who have crystallised human muscle phosphorylase-*a* and -*b*, found also two moles of pyridoxal phosphate per mole of phosphorylase-*b*, with a mol. wt. of 250,000, and four moles of pyridoxal phosphate per mole of phosphorylase-*a*, with a mol. wt. of 500,000. Lee (174, 175) has shown that pyridoxal phosphate is also a part of the molecule of purified potato phosphorylase which is electrophoretically homogeneous. The function of this pyridoxal phosphate residue, however, is still unknown.

Ions, and chiefly metal ions, are often activators, but α -amylase appears

to be the only glucosidase known in which a tightly bound metal ion, Ca^{++} , is an absolute requirement for activity (97, 295). In addition, Zn^{++} has been shown in *Bacillus subtilis* α -amylase to link two monomer-enzyme molecules to give a dimer (148, 270).

No purification of any nucleotide-transosylases has been achieved to date. In regard to the synthesis of heteropolymer of alternating units, it is not even known if one or two enzymes are implied.

SOME THERMODYNAMIC DATA

Equilibrium values of transosylation reactions give sufficiently precise indications of the energy of the osidic linkages and suggest the possible physiological role played by some of the transosylases.

In the synthesis of sucrose, the equilibrium value for sucrose phosphorylase (75) is:

$$\frac{[\text{Sucrose}][\text{P}]}{[\text{G-1-P}][\text{fructose}]} = 0.05 \quad \Delta F = +1700 \text{ cal.} \quad 25.$$

and for UDPG-sucrose transosylase (177):

$$\frac{[\text{Sucrose}][\text{UDP}]}{[\text{UDPG}][\text{fructose}]} = 5 \quad \Delta F = -1000 \text{ cal.} \quad 26.$$

This favourable equilibrium suggests that the UDPG enzyme is used by plants for the synthesis of sucrose, whereas sucrose phosphorylase may play a part in the catabolism of sucrose by *Pseudomonas saccharophila*.

Starting from the value of 4800 cal. for the C(1)—O—P linkage, from the sucrose phosphorylase equilibrium, it can be deduced that the value of the diosidic linkage of sucrose is 6600 cal.

The equilibrium attained by *B. subtilis* levansucrase acting on low molecular weight levan and glucose at 37° (242)

$$K = \frac{[\text{levan}_{n-1}][\text{sucrose}]}{[\text{levan}_n][\text{glucose}]} = 3.6 \cdot 10^{-2} \quad \Delta F = +2040 \quad 27.$$

gives for the fructofuranosido (2→6) fructose linkage an energy of about 4500 cal.

For maltose phosphorylase (98a).

$$K = \frac{[\text{maltose}][\text{phosphate}]}{[\beta\text{G-1-P}][\text{glucose}]} = 4.4 \quad \Delta F = -1000 \text{ cal.} \quad 28.$$

If the energy of the C(1)—O—P linkage is not essentially affected by α - β inversion, this implies a bond energy for maltose of about 4000 cal. From the equilibrium value of 4.3 found by Hestrin (135) with muscle phosphorylase transferring glucosyl from α -glucose-1-phosphate on glycogen, essentially the same value appears true for the α (1→4) glucosidic linkage in chains of glycogen. Trevelyan *et al.* (289), working with potato phosphorylase, found a ΔF of 1460 cal. and calculated an energy of 3400 cal. for the α (1→4)-glucosidic bond in the amylose chain.

There are no precise data for the evaluation of the α (1→6)-glucosidic

bond. Nevertheless, since *A. oryzae* transglucosidase, transgalactosidases, and dextrindextranase catalyze the transfer from 1→4 to 1→6 linkages, the equilibrium being favourable to the latter, Kalckar (155) assumes that these $\alpha(1\rightarrow6)$ linkages have a smaller energy.

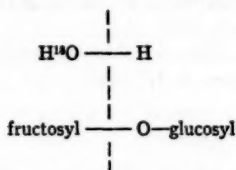
Robbins *et al.* (252) have shown that the transfer of a glucose unit from UDP glucose to glycogen should have a ΔF of -3000 cal. Conversion to glycogen would occur to an extent greater than 99 per cent.

From these data, it is clear that the nucleotide transosylases constitute the most favourable way for polysaccharide synthesis to take place. Starting from sucrose is another suitable way, and, in view of the manifold reactions which lead from sucrose to polysaccharides, hypotheses have been made concerning the possibility that other diosidic compounds, such as α,α' -trehalose, may play a part in polysaccharide synthesis (177).

NATURE OF THE TRANSFERRED RESIDUE

Cohn (49) was the first to show that the transferred radical is glycosyl, that is to say, the broken bond is between the C(1) of the transferred residue and oxygen. She used crystalline muscle phosphorylase, glycogen, and inorganic phosphate labeled with ^{18}O . At the end of the reaction, all the ^{18}O was found in inorganic phosphate and glucose phosphate. There was no loss to water which would have occurred had the P—O bond been broken. The same result was obtained with sucrose phosphorylase.

Koshland & Stein (167) studied sucrose cleavage by yeast invertase, a transfructofuranosylase, in presence of $\text{H}_2\ ^{18}\text{O}$. The glucose does not contain any ^{18}O ; that is, the fructose is liberated as fructosyl:



Using the $\text{H}_2\ ^{18}\text{O}$ technique, the same results were obtained with α - and β -amylase (120, 202) and β -glucuronidase (86).

SPECIFICITY

Bond-splitting specificity.—Because this review is limited to *trans*-O-glycosidases, the only bond which is split by these enzymes is the C—O bond. Thiogalactosides, for instance, are not split by β -galactosidases (209). There is, however, one exception: the β -glucosidase of *Stachybotrys atra* (151) splits β -phenylthiogluco-side. In general, thioglycosidases (116, 117) and N-glycosidases (194) are different enzymes with narrow bond-splitting specificity.

Another aspect of bond-splitting specificity is the retention or inversion of the glycosidic configuration. We have listed in Table I the principal data

known at present. From this table it is clear that the synthesis of the β linkage occurs chiefly by transfer from nucleotide-oses with inversion of the configuration. The α -D-configuration of the nucleotide-oses is established with reasonable certainty from the known pathway leading from UDPG to the other nucleotide-oses.

Glycosyl-transferred specificity.—Glycosyl-transferred specificity is usually narrowly delimited. With sucrose phosphorylase, the α -D-glucosyl moiety of α -D-glucose-1-phosphate cannot be replaced by α -D-galactosyl, α -D-mannosyl, α -D-xylosyl, or α -L-glucosyl (124).

TABLE I

SPLITTING OF THE GLYCOSIDIC BOND WITH RETENTION OR INVERSION
OF CONFIGURATION BY THE TRANS-O-GLYCOSYLASES

Inversion	Retention
β -Amylase	α -amylase
Taka-maltase	Almost all glycosidase transosylases
<i>B. macerans</i> cycloamylase	Levansucrase
Maltose phosphorylase	Dextransucrase
Cellobiose phosphorylase	Sucrose phosphorylase
UDPG $\rightarrow\beta$ (1-3) glucan transosylase	Muscle and amylophosphorylases
UDPG \rightarrow cellulose transosylase	UDPG \rightarrow glycogen transosylase
UDP-glucuronic acid \rightarrow glucuronides transosylase	—
UDPG $\rightarrow\beta$ -glucosides transosylase	—
UDP-N-acetylglucosamine \rightarrow chitin transosylase	—
UDP-glucuronic acid } \rightarrow hyaluronic acid	—
UDP-N-acetylglucosamine } transosylase	—
UDP-glucuronic acid } \rightarrow Type III pneumococcus	—
UDPG } transosylase	—

No changes may occur at C(2), C(3), or C(4) of the β -galactosyl residue without interfering with the action of the β -galactosidase of *E. coli*. The CH_2OH at C(5), however, can be replaced by CH_3 (β -D-fucoside) or H (α -L-arabinoside) (301). Highly purified *B. subtilis* levansucrase may hydrolyse α -D-glucosido- α -L-sorbofuranoside and α -D-glucosido- β -D-xyloketo-furanoside, thus allowing at the C(5) position of the fructofuranosyl residue an inversion of position of the CH_2OH or its replacement by H (69).

Donor specificity.—Precise indications may be obtained concerning the structural requirements needed for a radical to act as donor with an enzyme when this enzyme participates in exchange reactions. From the pioneer work of Hassid & Doudoroff (124) on sucrose-phosphorylase, it can be seen that this enzyme requires a donor radical linked with glucose possessing an hydroxyl adjacent to an acetal group in the *cis* position and in the same direction as the $-\text{OH}$ at C(2) of the α -glycopyranosyl rest (111). Whether phos-

phate binds at the same site as ketosyls is still uncertain. With levansucrase the disposition of the —OH at C(2) and C(3) found in glucose has to be respected (237). Some donor specificities are probably still more restricted; for instance, those of Q-enzyme and dextransucrase. With the maltose phosphorylase, although there is no exchange reaction, glucose may be replaced by xylose only; this observation implies a narrow specificity (98a).

In the cases of hydrolases, however, acyl- or arylglucosides, as well as oligosaccharides, act as substrates. The donor radical may vary widely. Moreover, it is difficult, to distinguish between donors and acceptors. Very often interchanges are possible, but this is not invariably true: transfer of glucuronosyl residue occurs from aryl- or alicyclic glucuronides to aliphatic alcohols and to glycols but not to phenols or to alicyclic alcohols (98).

Acceptor specificity.—For most of the transosylases, the normal acceptors are water, primary alcohols, phenols, or the primary or secondary alcohol groups of a simple sugar or a glycosidic chain. Many glycosidases-transosylases, such as most of the β -glucosidases and β -galactosidases, are capable of using each of these potential acceptors.

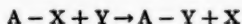
The transfructofuranosylases, invertases from yeast and from molds, transfer to water, to primary alcohols, or to the primary alcohol group of carbohydrates. But mold invertases transfer preferentially to C(1) of the fructosyl moiety of sucrose and yeast invertase to the C(6) of fructosyl and glucosyl (83).

Acceptor specificity is more limited for polysaccharide-synthesising enzymes. Phosphorylase requires a specific primer, as does UDP-glycogen transosylase, and amylomaltase, amylosucrase, *Bacillus macerans* amylase, and D-enzyme transfer solely to the C(4) of non-reducing glucose.

The data concerning two polysaccharide-synthesising enzymes are rather unusual. Dextranucrase and levansucrase produce chiefly high molecular weight dextran or levan (mol. wt. $> 10^6$). Many simple sugars and oligosaccharides act as acceptors for dextranucrase. In the same way, levansucrase transfers fructosyl residues to water, to the 6 position of fructose at the end of growing chain of levan, to the C(1) of the fructosyl moiety of sucrose, to C(6) and C(1) of free fructose, and to C(2), C(3), and C(6) of free glucose (89). In these two cases, small chains of dextran or levan have a very high affinity for the enzyme and are powerful activators of the reaction. For levansucrase, the K_m for sucrose is $5 \cdot 10^{-2} M$ but the K_A for levan is 3 to $6 \cdot 10^{-3}$. It appears that the normal activity of the enzyme seems to be determined by the ability of primer molecule to bind efficiently to the enzyme (69).

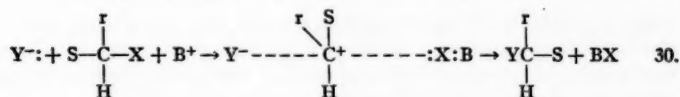
MECHANISM OF TRANSOSYLATION REACTION

Organic chemistry has shown that in the substitution reaction



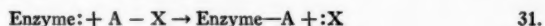
29.

the replacement of a group X by a group Y on a primary or secondary saturated carbon occurs by the following mechanism

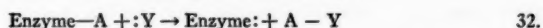


Y, with its unshared pair of electrons, brings about a nucleophilic attack on the carbon atom, and B, by pulling the electrons of X, an electrophilic attack.

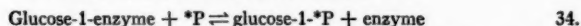
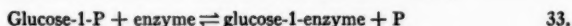
Koshland (165) proposed three main schemes to account for the known facts of the enzymic transfer reaction. The first was the single displacement mechanism. The substrate AX and the reactant Y were fixed on the enzyme's surface, Y attacking A from a position opposite X. This mechanism implies an inversion of the glycosidic bond and was proposed for the maltose phosphorylase reaction. The second, prompted by the glycosyl-enzyme hypothesis, was the double displacement mechanism. There is a preliminary attack on the substrate by a nucleophilic group on the enzyme's surface with splitting of the AX bond and the formation of a covalent bond between the enzyme and the radical to be transferred:



Then the enzyme-substrate intermediary is attacked by Y:



There is a double inversion which finally results in retention of the original configuration. This scheme is supported by the exchange reactions; for instance, the exchange of phosphate by sucrose-phosphorylase (75)



The third scheme was the frontal displacement mechanism. The substrates are fixed on the enzyme's surface in such a way that Y may attack on the side on which X is attached; i.e., the angle Y-AX is acute. With this mechanism, the original configuration is also retained.

This third possibility is in accord with the view of Jermyn (152), who thinks that the fixation of an acceptor molecule on its binding site is necessary before any reaction occurs. This hypothesis, which implies a ternary complex, is strongly supported by results obtained in kinetic studies of the β -glucosidase of *Slachybotrys atra*. Stringer & Tsuchiya (272) gave a similar interpretation of the activation of dextranucrase by α -methylglucoside.

The exchange reaction does not imply absolutely the existence of a glycosyl-enzyme compound. The only thing required is that the glycosyl residue is fixed in such a way that its life may be long enough to allow time for an exchange of the donor residue at its binding site. Without making any hypothesis concerning the nature of the bond between enzyme and fructosyl, Feingold (88) has speculated about the donor site of the three transfructofuranosylases, yeast invertase, mold invertase, and levansucrase. With yeast invertase, there is no exchange reaction. Feingold assumes that there is no specific binding site for the glucose moiety of sucrose. With levansu-

crase, reversibility of the reaction occurs, so that the glucose site is very stable. Mold invertase is a special case because an exchange reaction is observed only if sucrose is present; Feingold, in this case, supposes that a plastic site is formed to accept sucrose and that it has a short life.

Mayer & Larner (202), comparing the action of α - and β -amylase, suggest a similar first step in the reaction, the formation of a C_1 carbonium ion, followed by a stereospecific hydration which is dependent on the enzyme surface. Thoma & Koshland (285), however, found a rigorously quantitative inversion in β -amylase and concluded that no carbonium ion can be formed and that the simplest mechanism that explains the facts is a single displacement mechanism in which the approach of water is guided by the enzyme.

Koshland (166, 168), from an analysis of the results of inhibition in enzymic transfer reactions, thinks that key-lock theory of enzyme substrate specificity does not answer every question, and he suggests the "induced fit" theory which has three main postulates: (a) a considerable change is induced in the protein's geometry by the substrate when the latter is fixed to the binding site; (b) a precise orientation of the catalytic groups is needed for enzyme activity; and (c) the substrate induces this proper orientation by the modification produced in the protein's geometry. Competitive inhibition of β -amylase with cyclodextrins and with the internal part of its normal substrate, amylopectin, supports this "induced fit" theory (284). As an extension of the Jermyn theory, it may be said that in some cases, specific acceptor molecules act also to induce proper geometrical configuration. This occurrence may be true for such enzymes as levansucrase or dextranase.

GROUPS AT THE ACTIVE SITE

Using the inhibition by heavy metals or specific inhibitors, such as *p*-mercuribenzoate, intervention of SH groups in the enzymic activity has been found in many transosylases, including β -amylases, myrothecium cellulase, amylo-1-6-glucosidase, β -glucosidase from *S. cerevisiae* and *St. atra*, yeast invertase, β -glucuronidase, and β -galactosidase from *E. coli*. These results have been confirmed for *S. cerevisiae* β -glucosidase with competitive protection by the substrate against inhibition by SH reagents (76). Thoma & Koshland (286) found also that cyclohexa-amylose, a competitive inhibitor of β -amylase, protects this enzyme against inhibition by iodacetamide. As for β -galactosidase from *E. coli*, the quantitative relation between the binding of SH groups by *p*-mercuribenzoate and the decrease of the activity is good proof (301).

The pH activity curve of an enzyme gives an indication of the pK of the groups at the active site. For β -galactosidase from *E. coli*, Wallenfels found indications for two groups, one with a pK of 5.8 to 6.2 and the other with a pK of 7.8 to 8.6. The latter is identified with an SH group, the former is supposed to be an imidazol ring (306).

Thoma & Koshland (286), from the plot V_m/pH for β -amylase deduced the presence of two active groups, one with a pK of 4.3, the other with a pK of 7.1, most probably a carboxylic group and an imidazol group, re-

spectively. They think that at least three groups are involved in β -amylase activity: a carboxylic group, an imidazol ring, and an SH group.

Neely (214) found two pK values from the pH activity curve of dextransucrase: pK_a between 4.9 and 5.1 and pK_b between 5.5 and 5.7. The former is attributed to a nucleophilic carboxyle, the latter to an imidazol ring which acts as electrophilic group. Neely (215) confirmed the participation of the imidazole group of histidine by photo-oxidation experiments which led to the inactivation of dextransucrase. Concomitant destruction of histidine has been proven. The presence of the substrate during this photo-oxidation protects the activity of the enzyme and suppresses the destruction of histidine.

SYNTHESIS OR BREAKDOWN OF LONG CHAINS

The postulation by Cleveland & Kerr (48) and Swanson & Cori (281) of a single-chain mechanism for the breakdown of amylose by β -amylase arose from the observation that the amylose which remains at the intermediate stage of hydrolysis appears to be identical to original substrate. Bailey & Whelan (17), using low molecular weight amylose, established that this observation is not entirely true and that multichain hydrolysis occurs at least in part. Bailey & French (16), by the kinetic study of hydrolysis of end-labeled amyloextrins, found evidence of a "multiple attack" by the enzyme; i.e., during the life of an enzyme substrate complex about four cleavages occur.

The same problem arises concerning the synthesis of chains, and Stacey (269) has proposed the single-chain process for the formation of dextran. The results of Bovey (37), obtained by examination of growing dextran with light scattering, showed that without acceptor dextransucrase produces high molecular weight dextran which is homogeneous from the beginning to the end of the reaction, thus, suggesting a single-chain mechanism. With acceptors, however, low molecular weight dextrans are formed which are very heterogenous; and, in this case, a multichain process would seem to occur. It is interesting to note that Bovey has found a net increase in the molecular weight of the highly polymerised dextran after the total consumption of the sucrose. It has not been established whether this phenomenon is attributable to the same enzyme that synthesises dextran from sucrose.

With levansucrase it has been found also that in presence of primers highly polymerised levans and low molecular weight levans are both formed. The latter may be degraded to sucrose by levansucrase acting in the back-side direction, but the former are inert (70a). Indications have been obtained, however, of the binding of the enzyme to high molecular weight levans (242).

On the basis of the known data it may be said that the high affinity of primer of increasing molecular weight for the enzyme can explain the formation of large molecules on the basis of a dissociable enzyme-product complex by a multichain process (69). At an unknown level of polymerisation, however, the enzyme product complex behaves as an undissociable complex, and the synthesis can occur according to a single-chain mechanism.

ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES FROM SINGLE SUGARS

This synthesis proceeds from reversibility of the hydrolysis reaction. The occurrence of such a reaction was shown by Bourquelot & Bridel in 1912; they demonstrated the synthesis of methyl- β -D-glucopyranoside by the action of the β -glucosidase from emulsin on methanol and D-glucose and the formation of gentiobiose (35, 36). Peat, Whelan & Hinson (234) allowed β -glucosidase from emulsin to act for several weeks on glucose at a concentration of 60 per cent and obtained a series of β -diglucosides: β -trehalose (1 \rightarrow 1'), sophorose (1 \rightarrow 2), laminaribiose (1 \rightarrow 3), cellobiose (1 \rightarrow 4), gentiobiose (1 \rightarrow 6), and higher oligosaccharides. Clancy & Whelan (46) obtained by the action of an α -galactosidase of *Saccharomyces carlbergensis* the formation of several oligosaccharides; the main product was 6-O- α -D-galactopyranosyl-D-galactose. Wallenfels (301) obtained the synthesis of oligosaccharides with β -galactosidase of *E. coli*, acting on dilute solution (2 to 5 per cent) of a mixture of glucose and galactose, by circulating the mixture on a carbon-celite column.

MISCELLANEOUS

 α -AMYLASE

Properties of α -amylases have been reviewed by Fischer & Stein (97). Their use in structural examination of starch has been exposed by French (100a), and Whelan and co-workers (32, 253, 299, 300) have re-examined the action of salivary α -amylase on amylose, amylopectin, glycogen, and phosphorylase limit dextrins of glycogen and of amylopectin. Ikenaka (147), after treatment by fluorodinitrobenzene and *p*-phenylazobenzoyl chloride suggests that tyrosine is absolutely required for Taka- α -amylase action. The substitution by acetylation of one-half of the amino groups in α -amylase of hog pancreas and in Taka-amylase leads to inactivation (248). Terminal amino acids have been investigated in α -amylase of *A. oryzae* (146, 292, 293) and of *B. subtilis* (278). Immunological experiments have shown that hog pancreatic, salivary, and seric α -amylase are immunologically similar but that hog liver α -amylase is completely different (192, 193). Yoshida & Tobita (322), using a leucine-requiring mutant of *B. subtilis*, have shown the α -amylase is produced only at the end of the growing phase. At this stage, incorporation of ^{14}C -leucine leads to a greater specific radioactivity in the cellular proteins than in α -amylase. They concluded that α -amylase was formed from a precursor. Yoshida *et al.* (323) have isolated the precursor on a diethylaminoethyl cellulose column from the bacterial protein before the formation of α -amylase. The precursor is antigenically similar to α -amylase but has no activity. Its molecular weight is 43,000 instead of 49,000 for α -amylase. The *B. subtilis* α -amylase would be an unique polypeptidic chain with the N-terminal sequence: $\text{H}_2\text{N-Val-Asp-Gly-Glu-(Ser-Ala-Val)(Leu-Leu-Thr-Gly-Lys)}$ and the carboxyl terminal sequence: $(\text{Ala-Tyr-Val-Leu})\text{-Asp-(NH}_2\text{)OH}$. The biosynthesis of *B. subtilis* α -amylase is stimulated by alkyldiamines or polyalkylamines (324). Ulmann *et al.* (294) have shown that

α -amylase is synthesized in a mitochondrial preparation of pigeon pancreas also from a precursor. Schiff, Eisenstadt & Klein (87, 263) did not find intervention of a precursor in the synthesis of α -amylase in *P. saccharophila*.

β -AMYLASE

French has given a general survey of the properties of the β -amylases (100) and of their contribution to the elucidation of starch structure (100a). Recent progress in the knowledge of the mechanism of action of β -amylases has been already quoted in this review (p. 370).

OTHER GLYCOSIDASES

Larner (172) has reviewed the properties of the main α - and β -glucosidases. Rosenfeld (256) has found in liver homogenate an enzyme of the glucoamylase type, which splits glucose units from glycogen or amylopectin; the residual dextrans are high iodine-staining polymers called γ -dextrans. The same preparations also split terminal glucose residues from polyglucosides of the dextran type (258). The authors were unable to separate the two activities with fractionation techniques or to differentiate them by inhibitors. But the $\alpha(1\rightarrow6)$ -polyglucosidase activity of rabbit liver is practically absent during the spring and summer months, whereas the $\alpha(1\rightarrow4)$ activity is retained.

Myrbäck (212) has given a comprehensive review of invertases, particularly yeast invertases. Known facts on pectinases may be found in three fully documented reviews (71, 72, 96).

Howard *et al.* (141) found in two rumen bacteria two xylosanases: an endoxylanase which does not split xylobiose or xylotriose, and a β -xylobiase which splits xylobiose and also acts on β -phenyl xyloside. Fucosidase may be obtained from *Microcystus poryfera*, an organism which produces fucane sulfate (310). In mammals an α -L-fucosidase occurs in rat liver and epididymis, which is inactive on α -D-fucoside, but the α -L-fucosidase from *Patella vulgata* is not devoid of action on α -D-fucoside [Levvy (184)]. The so-called H-enzyme from *Trichomonas foetus* appears to be an α -L-fucosidase also (309). It is interesting to note that α -L-galactosidase has been used with great success to determine the structure of the specific terminating part of the B blood group substance (328).

HYALURONIDASES

Hyaluronidases have been divided by Meyer *et al.* (203, 204) in three classes: testicular hyaluronidase, an endoglucosaminidase which gives as a final product a disaccharide of the type glucuronosido-N-acetyl glucosamine; bacterial hyaluronidase, also an endoglucosaminidase which gives desaturated disaccharides $\Delta 4$ -5-glucuronoseenido-N-acetylglucosamine; and leech hyaluronidase, an endoglucuronidase. Testicular and bacterial hyaluronidase do not display a very strict specificity; they hydrolyse hyaluronic acid and also the chondroitin sulfates, splitting N-acetyl-glucosamine and N-acetyl galactosamine. The production of desaturated disaccharide by bacterial

ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES FROM SINGLE SUGARS

This synthesis proceeds from reversibility of the hydrolysis reaction. The occurrence of such a reaction was shown by Bourquelot & Bridel in 1912; they demonstrated the synthesis of methyl- β -D-glucopyranoside by the action of the β -glucosidase from emulsin on methanol and D-glucose and the formation of gentiobiose (35, 36). Peat, Whelan & Hinson (234) allowed β -glucosidase from emulsin to act for several weeks on glucose at a concentration of 60 per cent and obtained a series of β -diglucosides: β -trehalose (1 \rightarrow 1'), sophorose (1 \rightarrow 2), laminaribiose (1 \rightarrow 3), cellobiose (1 \rightarrow 4), gentiobiose (1 \rightarrow 6), and higher oligosaccharides. Clancy & Whelan (46) obtained by the action of an α -galactosidase of *Saccharomyces carlbergensis* the formation of several oligosaccharides; the main product was 6-O- α -D-galactopyranosyl-D-galactose. Wallenfels (301) obtained the synthesis of oligosaccharides with β -galactosidase of *E. coli*, acting on dilute solution (2 to 5 per cent) of a mixture of glucose and galactose, by circulating the mixture on a carbon-celite column.

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hyaluronidases, which has been confirmed with *Flavobacterium* enzymes (140, 188), is still disputed with *Proteus vulgaris* enzyme. Dodgson, in the action of *Proteus vulgaris* "chondroitinase" on hyaluronic acid or on chondroitin sulfate found only normal saturated disaccharides (73, 74). Nakada and co-workers (201, 213), in independent work, obtained the $\Delta 4$ -5-unsaturated disaccharides, and Suzuki (280), using the *Proteus vulgaris* enzyme on chondroitin sulfate A, B, and C, found three $\Delta 4$ -5-unsaturated disaccharides, one monosulfate, and two disulfates. In *Flavobacterium* adapted on chondroitin sulfate there is an enzyme which splits the desaturated disaccharides to give free N-acetyl glucosamine and a keto acid (188, 189). Extracts of *Coccobacillus* (Chase) hydrolyse keratosulfate by the action of two enzymes: an exo- β -galactosidase and an exo- β -glucosaminidase (254).

β -GLUCOSAMINIDASE

In addition to testicular and bacterial hyaluronidases, there are three types of β -glucosaminidases. The first is the arylglucosaminidase found in mammalian tissues; it has been purified by Findlay & Levvy (93) from pig epididymis and by Woolen *et al.* (319) from ram testis. The enzyme acts by the same site on N-acetylglucosaminides and N-acetylgalactosaminides. The search for useful synthetic substrates was concluded with the use of 4-methyl umbelliferyl-N-acetyl glucuronosaminide (34, 173). The second is the chitinase, $\beta(1 \rightarrow 4)$ glucosaminidase. Chitinase from *Streptomyces* has been investigated by Jeumiaux (153) and Berger & Reynolds (30). These authors found three constituents: two true chitinases and one chitinbiase. This system, which resembles the β -glucosidases complex of cellulolytic organisms, has also been found in the crab *Carcinus maena* (191a). The third type of glucosaminidase is lysozyme. The properties of lysozyme have been recently reviewed by Jolles (154). Berger & Weiser (31) found that lysozyme is a β -glucosaminidase, most probably is specific for the $(1 \rightarrow 4)$ linkage, and hydrolyses chitin. Salton & Ghuyssen (259, 260) and Perkins (243) confirmed the fact by isolating, from digests of *Micrococcus lysodeikticus* cell walls, a di- and a tetrasaccharide with the following structures: 6-0- β -N-acetyl glucosaminyl-N-acetyl muramic acid and 0- β -N-acetyl glucosaminyl $(1 \rightarrow 6)$ -0- β -N-acetyl muraminyl $(1 \rightarrow 4)$ -0- β -N-acetyl-glucosaminyl $(1 \rightarrow 6)$ - β -N-acetylmuramic acid. Hamaguchi *et al.* (122) found action of lysozyme on glycol-chitin.

β -GLUCURONIDASE

The properties of exoglucuronidases have been reviewed by Levvy & Marsh (186, 187). They have been purified from extracts of preputial glands of female rats and found widely distributed in the body (52, 185). A very purified preparation of β -glucuronidase from *Helix pomatia* has been obtained by Alfsen & Jayle (4), and its specificity for the hydrolysis of several steroid glucuronides has been investigated (3).

The leech hyaluronidase is an endoglucuronidase (203). Torriani & Pappenheimer (288a) have found that the inducible depolymerising enzymes

from *Bacillus palestris* that act on the Type III and on the Type VIII pneumococcal polysaccharides are $\beta(1 \rightarrow 4)$ endoglucuronidases, but their specificity differs. The linkage to be split must be preceded by a glucose unit linked in $\beta(1 \rightarrow 3)$ in the case of the Type III depolymerase or followed by a glucose unit linked in $\beta(1 \rightarrow 4)$ in the case of the Type VIII depolymerase.

NEURAMINIDASE

The so-called receptor-destroying enzyme has been shown by Klenk *et al.* (159) to split N-acetylneuraminic acid. Kuhn & Brossmer (169, 170) and Gottschalk (112) define its action as the hydrolytic cleavage of the glucosidic bond joining the keto group of neuraminic acid to D-galactose or D-galactosamine and possibly to other sugars. The properties of neuraminidase have been reviewed by Gottschalk (113, 114) who has also given an account of the structure and occurrence of sialic acid (114a). Neuraminidase has been crystallised from *Vibrio cholerae* by Ada & French (2) and Schramm & Mohr (265). Gottschalk *et al.* (115) have shown that neuraminidase splits sialic acid residues from follicle-stimulating hormone. This elimination of the α -ketosidically linked sialic acids led to an inactivation of 97 per cent.

The product, N-acetylneuraminic acid, is a competitive inhibitor for the neuraminidase from influenza virus. The K_m for N-acetylneuraminyllactose as a substrate is 6.10^{-4} M, and the K_I for N-acetyl muramic acid is 5.10^{-3} M (308).

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WATER-SOLUBLE VITAMINS, PART I^{1,2}

(ASCORBIC ACID, BIOTIN, INOSITOL, NICOTINIC ACID, PYRIDOXIN GROUP)

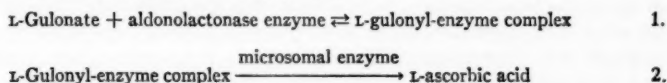
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L-ASCORBIC ACID

Biosynthesis.—Chatterjee *et al.* (1) have presented a detailed investigation on the mechanism of synthesis of ascorbic acid in animal tissues; they summarize the experimental basis for the scheme of ascorbic acid biosynthesis presented in a previous review (2). Inhibition by flavin inhibitors of a soluble enzyme preparation catalyzing the conversion of L-gulonolactone into L-ascorbic acid suggested that this reaction may be mediated by a flavoprotein. The involvement of a lipid cofactor in the conversion of L-gulonolactone into L-ascorbic acid by goat liver microsomes was indicated (3). α -Tocopherol was the most effective substance tested in restoring the activity of lipid-extracted microsomes. Chatterjee *et al.* (4) submitted evidence for the production of hydrogen peroxide in the aerobic conversion of L-gulonolactone to 2-keto-L-gulonolactone by soluble liver microsomal preparations. Further studies on the mechanism of conversion of D-glucuronolactone and of L-gulonolactone into L-ascorbic acid by the liver microsomes of the rat and goat and the kidney microsomes of the chick, as well as by soluble enzyme preparations obtained from these microsomes, have been presented (5).

Isherwood *et al.* (6) have made a detailed analysis of the conversion of L-gulonolactone into L-ascorbic acid by isolated rat liver mitochondria and microsomes. Microsomes were four times as active as mitochondria. The formation of L-ascorbic acid from L-gulonolactone by homogenates involved an aldololactonase present in the supernatant fraction. No evidence was obtained that 3-oxogulonolactone was an intermediate in this conversion, and the following reaction sequence was suggested:



A study of the synthesis of ascorbic acid in isolated enzyme systems by Chang & Tung (7) yielded results very comparable to those discussed in this

¹ The survey of the literature pertaining to this review was concluded in October, 1960.

² The following abbreviations are used: CoA for coenzyme A; NAD for nicotinamide-adenine dinucleotide; NADase for nicotinamide-adenine dinucleotidase; NADP for nicotinamide-adenine dinucleotide phosphate.

section. Feinberg & Caputto (8) have shown that the synthesis of ascorbic acid from L-gulonolactone by a suspension of liver microsomes from the rat or a soluble preparation obtained from these microsomes was stimulated by hydrogen peroxide. The synthesis of ascorbic acid in systems activated by hydrogen peroxide was decreased in liver preparations from vitamin E-deficient rats. Loewus *et al.* (9) have studied the synthesis of ascorbic acid from D-glucose-2-C¹⁴ in the intact rat. Ascorbic acid was recovered from the liver, the presumed site of synthesis. Their results confirmed previous observations which indicated that ascorbic acid is synthesized from D-glucose in the mammalian organism by way of reactions that lead to inversion of the hexose carbon chain.

Carpenter *et al.* (10) have confirmed previous observations that liver preparations from vitamin E-deficient rats and rabbits have an inhibited rate of ascorbic acid synthesis. They have now demonstrated an increased lipid peroxide-forming activity in such liver preparations from vitamin E-deficient animals. Both phenomena may be related since all factors that reactivate ascorbic acid synthesis in these preparations Co⁺⁺, Mn⁺⁺, Ce⁺⁺⁺, ethylenediaminetetra acetate, dithizone, tocopherol, diphenyl-*p*-phenylene-diamine, and Santoquine (7-chloro-4-(4-diethylamino-1-methylbutylamino)-3-methylquinoline) also prevent peroxide formation. The bearing of these findings obtained with an *in vitro* system upon the biochemical function of tocopherol *in vivo* must remain in doubt until further information on a physiological relationship between vitamin E and ascorbic acid becomes available. Data presented on the peroxide content of tissues from vitamin E-deficient rats and rabbits were not contributory. A later study also on liver preparations from vitamin E-deficient rats (11) demonstrated the formation of thiobarbituric acid-reacting materials. At least two such reacting materials were produced; the most abundant appeared to be malonaldehyde, implicated previously as a result of lipid peroxidation. It appears that gulonolactone oxidase, either in the intact microsome or after solubilization, is inhibited by some factor related to the process of formation of malonaldehyde but not by malonaldehyde itself. The physiological role of vitamin E in these reactions requires further clarification.

Burns *et al.* (12) have demonstrated the stimulatory effect of a variety of drugs on the biosynthesis of L-ascorbic acid in the albino rat. The mechanism by which drugs stimulate ascorbic acid synthesis is not known, but renal factors and drug glucuronide formation apparently are not involved. Barbitol and chlorobutanol (Chloretone) enhance the synthesis of free D-glucuronic acid and L-gulonic acid, and the authors suggest that the drug effect on ascorbic acid formation results from increased metabolism of glucose through the glucuronic acid pathway. Evans *et al.* (13) have shown experiments *in vivo* with the rat that D-galactose-1-C¹⁴ is a better precursor of D-glucuronic acid, L-gulonic acid, and L-ascorbic acid than D-glucose-1-C¹⁴. D-Galactose-1-C¹⁴ was also converted to D-glucuronic acid and L-gulonic acid in rat liver homogenates. These results are in accord with a mechanism of L-ascorbic acid

biosynthesis which involves the uridine nucleotides. The striking effect of barbital in stimulating the metabolism of D-galactose to D-glucuronic acid, L-gulonic acid, and L-ascorbic acid was also noted. Ulanova & Yanovskaya (14) noted an increase in the ascorbic acid concentration of a number of organs of the white rat following inhalation of methylene chloride.

The inhibitory effect of the alkaloid lycorine upon ascorbic acid synthesis in the rat has been described previously. Yamaguchi (15) has determined that lycorine inhibits ascorbic acid synthesis in the chick embryo and in a germinating bean. It also antagonizes the accelerating effect of chlorobutanol upon ascorbic acid biosynthesis. The locus of action of lycorine was also studied.

Ascorbigen (the bound form of ascorbic acid) is an indole derivative containing an indole moiety with a carbon skeleton identical with that of tryptophan. In a recent study with DL-tryptophan-3-C¹⁴, Kutáček *et al.* (16) concluded that tryptophan can be utilized in *Brassica oleracea* L. for the synthesis of ascorbigen.

Functions.—Abt *et al.* (17) have investigated the ascorbic acid, collagen, and hexosamine distribution in scars produced in guinea pigs on various ascorbic acid dietary levels following wounding by abdominal incision. Fractionation of tissues that comprise the scar revealed that the highest concentration of ascorbic acid was present in the connective tissue. Determinations of collagen (based on hydroxyproline analyses) and hexosamine content of abdominal scar tissue and of tissue produced in carrageenin granuloma revealed no differences between scorbutic and normal animals. It is difficult to reconcile the failure to observe a decreased collagen content with the histological evidence that the formation of connective tissue fibres is impaired in scurvy. These workers (18) have also noted that in the guinea pig abdominal wounds which had been allowed to heal for a long period could be ruptured after scurvy had been produced. The possible non-specific effects of inanition were not investigated. The ascorbic acid which accumulated in scar tissue immediately following wounding persisted for long periods of time. Jasinski & Lotmar (19) have confirmed that the incorporation of S³⁵ into the chondroitin sulfate of the cartilage is diminished in scorbutic guinea pigs.

In photosynthesis, Marré *et al.* (20) believe that the ultimate electron acceptor in ascorbic acid photo-oxidation is the oxidizing agent produced in photolysis of water and that in illuminated whole chloroplasts an ascorbic-monodehydroascorbic acid system is continuously subjected to two opposite processes, one of oxidation and the other of reduction, both strictly depending on the photolysis of water.

Mazur *et al.* (21) have studied the mechanism of plasma iron incorporation into hepatic ferritin. The release of iron from its linkage to the plasma iron-binding protein, transferrin, and its subsequent incorporation into hepatic ferritin is dependent on oxidative metabolism of the liver cell, specifically for the continued synthesis of ATP. In addition to ATP, ascorbic acid is also required for the incorporation reaction. A mechanism to explain

the roles of ATP and ascorbic acid envisioned the formation of a complex involving two moles of ATP, one mole of ascorbic acid, and the iron-transferrin protein of plasma. The function of ascorbic acid presumably involves its action as a reducing agent, converting transferrin iron from the ferric to the ferrous state. Lockhead & Goldberg (22) have implicated ascorbic acid and glutathione in the transfer of iron from liver ferritin and hemosiderin, and from rat and human siderophilin, to protoporphyrin in the biosynthesis of heme.

Breslow & Lukens (23) have re-examined the model hydroxylating system which consists of ascorbic acid, iron salts, ethylenediaminetetra acetate, buffer, and either oxygen or hydrogen peroxide, which can hydroxylate various aromatic compounds. Interest in this system stems from the possibility that ascorbic acid may be involved in similar reactions *in vivo*. In the hydrogen peroxide system, it is proposed that ascorbic acid functions to reduce the ferric ion produced by the interaction of hydrogen peroxide with ferrous ion; in the oxygen system, ascorbic acid serves in addition as the source of the required hydrogen peroxide via an iron-catalyzed auto-oxidation. Acheson & Hazelwood (24) have noted that the proportions of mono- and dihydroxybenzoic acids formed in the hydroxylation of benzoic acid by oxygen in the presence of ascorbic acid, ferrous sulfate, and ethylenediaminetetracetic acid are consistent only with a homolytic substitution. The presence of free radicals in the hydroxylation mixture was confirmed by polymerization experiments. Grinstead (25) has studied the kinetics of the oxidation of ascorbic acid by hydrogen peroxide in the presence of the iron chelate of ethylenediaminetetra acetic acid and the direct reduction of the ferric chelate by ascorbic acid.

In their studies on the relationship of scurvy to carbohydrate metabolism, Banerjee & Biswas (26) have investigated the urinary excretion of pyruvic acid, α -ketoglutaric acid, and oxaloacetic acid in normal and scorbutic guinea pigs before and after the feeding of citric, succinic, and malic acids. The abnormal excretory pattern of these Krebs cycle intermediates was restored with insulin treatment. Banerjee & Singh (27) found further evidence for a disturbance of citric acid metabolism in scorbutic guinea pigs. Insulin treatment was again effective in ameliorating this metabolic defect which is presumed to be at the level between citric and α -ketoglutaric acids and not in the aconitase system. Banerjee & Bal (28) have studied various aspects of carbohydrate and sterol metabolism in normal and scorbutic monkeys. Disturbances were reminiscent of those noted in scorbutic guinea pigs. Lin & Cohen (29) have shown that scorbutus in guinea pigs plus pentobarbital treatment raises the *in vivo* creatine phosphate level and interferes with the turnover of creatine phosphate and ATP. This synergistic effect of pentobarbital depression and ascorbic acid deficiency represents another example of what appears to be a definitely antagonistic relationship between ascorbic acid and pentobarbital on certain metabolic processes.

It has long been known that the feeding of L-tyrosine to premature in-

ants and scorbutic guinea pigs, monkeys, and man causes an excretion of *p*-hydroxyphenylpyruvic acid which can be prevented by ascorbic acid. Important contributions to the understanding of the mechanisms involved in this effect have been made by Knox & Goswami (30) and Zannoni & La Du (31). Tyrosine administration causes the excretion of *p*-hydroxyphenylpyruvic acid by increasing the activity of tyrosine- α -ketoglutarate transaminase and decreasing that of *p*-hydroxyphenylpyruvic acid oxidase. The inhibition of the oxidase can be prevented by administration of ascorbic acid and by other compounds, such as 2,6-dichlorophenolindophenol, D-isoascorbic acid, and D-glucoscorbic acid.

Utilizing purified semisynthetic diets, Beaton *et al.* (32) have reinvestigated the effects of scurvy in the guinea pig upon thyroid activity. Their data indicated that thyroid activity was increased in scorbutic animals and that this increase was associated with an abnormal metabolism of tyrosine.

Barkhan & Howard (33) reported a depression in the coagulation reaction of blood from scorbutic guinea pigs which was evident at the prothrombic and thromboplastic levels. The exact nature of the defect was not established. The activation of blood coagulation by kaolin and glass surfaces was investigated in normal and scorbutic guinea pigs (34). A defective reaction to glass contact and a prolonged kaolin-clotting time were noted in scurvy. In man, a defective ability to react to glass contact is associated with a congenital deficiency of Hageman factor or plasma prothrombin antecedent. Banerjee & Bal (35) have studied changes in bone marrow and blood picture in monkeys during progressive stages of scurvy. The anemia of scorbutic monkeys was normocytic, normochromic, and normoblastic in character. There was progressive leucopenia with diminution in number of neutrophils, lymphocytes, and monocytes and an increase in the number of eosinophils during development of scurvy. In well-controlled experiments, Constable (36) failed to note the development of anemia in guinea pigs with acute or chronic scurvy.

Géro *et al.* (37) have described a sparing action of small amounts of cabbage for vitamin C in the guinea pig which was manifested by an increased level of ascorbic acid in the adrenals and a diminution of scorbutic symptoms. The chemical nature of the factors in cabbage is unknown.

Neuman & Neuman (38) have reviewed the role of ascorbic acid in bone growth. Dadd (39) has determined the nutritional requirement of ascorbic acid for the normal development to the adult stage in two locusts, *Schistocerca gregaria* (Forskål) and *Locusta migratoria* L. The conclusion was drawn that in locusts ascorbic acid plays a role in the processes occurring at the moult.

Assessment of vitamin-C nutriture.—Dutra de Oliveira *et al.* (40) have described a simplified oral ascorbic acid tolerance test which is useful in assessing vitamin-C nutriture. This entails the measurement of serum ascorbic acid level following oral administration of ascorbic acid and is helpful in sustaining the clinical diagnosis of scurvy. Chadwick *et al.* (41) have utilized a simple method for one-dimensional chromatography to follow the course of

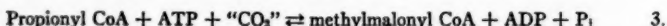
amino-aciduria in a patient with advanced scurvy. Tani (42) has recorded experiences with a saturation test for vitamin-C adequacy in human subjects. To obtain a true picture of the state of vitamin-C nutriture, the author recommended the performance of a second saturation test subsequent to the daily administration of ascorbic acid for one week. Utilizing the urinary excretion of oral test doses of vitamin C, Lester *et al.* (43) found that the tissue vitamin-C level of hospitalized alcoholics was lower than that of non-alcoholics. Lewis & Chiang (44) reported that the bound ascorbic acid level of serum and liver was not affected in the guinea pig by depletion of ascorbic acid or by variations in protein intake. The interpretation of these findings is clouded by the non-specificity of the analytical procedure used to determine bound ascorbic acid (45).

Determination.—Freebairn (46) has determined the superiority of a dilute ethylenediaminetetraacetic acid-trichloroacetic acid solution over metaphosphoric or oxalic acids as an extraction medium for ascorbic acid of plant material. Chalet & Chalet (47) measured ascorbic acid by agar diffusion which possesses certain advantages over titrimetric procedures. Booth & Constable (48) have reported a value for ascorbic acid in the iris leaf which is among the highest so far recorded for ascorbic acid in leaves. Of interest is the exceptionally low oxidase activity of iris leaf toward ascorbic acid.

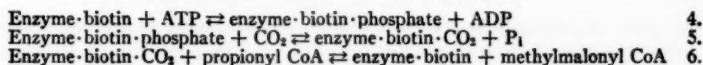
Metabolism.—Kanfer *et al.* (49) indicate that the major metabolic products of L-ascorbic acid metabolism in the rat kidney are L-xyloonic and L-lyxonic acids. An enzyme system capable of decarboxylating dehydro-L-ascorbic acid and 2,3-diketo-L-gulonic acid was partially purified from rat kidney, and the products of the reaction were identified as L-lyxonic and L-xyloonic acids.

BIOTIN

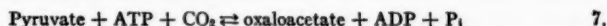
Interest in the metabolic function of biotin has continued during the past year. The CO₂ carrier role of biotin in diverse enzyme systems has been further demonstrated. Stadtman and co-workers (50) have indicated that biotin functions in the metabolism of *Propionibacterium shermanii* as a CO₂ acceptor in the conversion of methylmalonyl CoA to propionyl CoA. The participation of a CO₂-biotin-enzyme complex was envisioned in this reaction. The function of biotin in this case is analogous to its role in the transformation of β -methylglutaconyl CoA to β -methylcrotonyl CoA (51). The participation of biotin in the mechanism of action of propionyl carboxylase isolated from bovine liver mitochondria has been indicated by Halenz & Lane (52). This enzyme catalyzes the reaction:



The authors suggest the following reactions as a possible mechanism for the enzymatic carboxylation of propionyl CoA:



It will be recalled that Lynen and co-workers (51) have proposed the formation of an ADP~biotin·enzyme complex in the sequence of reactions involving the β -methylcrotonyl CoA carboxylase of *Mycobacteria*. Utter & Keech (53) have studied an enzyme system derived from avian and beef liver which catalyzes the over-all reaction:



CO_2 fixation was almost completely inhibited by avidin, and this effect could be nullified by the prior addition of biotin to the avidin. Swick & Wood (54) have indicated that in *Propionibacterium shermanii* biotin functions as a co-factor in the transcarboxylation reaction, yielding propionyl CoA and oxaloacetate from methylmalonyl CoA and pyruvate. They suggest that this reaction may well be mediated by a CO_2 ·biotin·enzyme complex of the type proposed by Lynen and co-workers (51).

A recent symposium (55) dealt with metabolic reactions involving biotin. Lardy has reviewed the historical development of this field. Swick & Wood have discussed the role of biotin in the transcarboxylation reactions of propionibacteria. Wakil has reviewed studies relating to the participation of biotin in fatty acid synthesis by purified fractions of avian liver, particularly its role in acetyl CoA carboxylase [cf. (56)]. Lynen has reported on the action of biotin as a biological CO_2 carrier in the β -methylcrotonyl carboxylase system of *Mycobacteria* and the highly significant identification of N-carbo-methoxybiotin methyl ester as the product formed when biotin serves as substrate in lieu of β -methylcrotonyl CoA (cf. 57). Kaziro and co-workers have presented evidence for the role of biotin in the propionyl CoA carboxylase of pig heart. Stern & Friedman (58) have indicated the function of biotin in the enzymatic carboxylation of butyryl CoA by a purified enzyme from ox liver.

The role of biotin as a biological CO_2 carrier is well-documented. Supporting evidence has been derived in large part from experiments utilizing the specific ability of avidin to bind and inactivate biotin. In some instances these data have been buttressed by the observation that the biotin content of the enzyme in question increases proportionately with further purification. An unequivocal elucidation of the role of biotin must, however, await isolation of the biotin intermediates involved in these enzymatic reactions, their chemical identification, and the demonstration of their biological activity in isolated *in vitro* systems. The work of Lynen and his co-workers represents a significant step in this direction.

Other metabolic functions of biotin are also under investigation. Rose (59) has produced evidence for the accumulation of free nicotinic acid and nicotinic acid adenine dinucleotide in the medium of yeast cells grown in the

presence of suboptimal levels of biotin. Nicotinamide in free or combined form was absent from these culture filtrates. The author suggests that the further metabolism of nicotinic acid into nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide into NAD³ is inhibited in biotin deficiency. A selective permeability of the biotin-deficient cells for these intermediates has not been excluded and remains as a possible explanation for the results obtained. Determination of the concentrations of these compounds in biotin-deficient cells would aid in the interpretation of these data. Moat & Nasuti (60) have reported on the role of biotin in the conversion of aminoimidazole to aminoimidazole carboxamide by *Saccharomyces cerevisiae*. Viable cells have been utilized in both of the foregoing studies, and, under these circumstances, it is difficult to determine whether biotin has a direct function as a coenzyme or plays an indirect role, perhaps in the synthesis of an enzyme. Ravel & Shive (61) had previously shown that a biotin deficiency in *Streptococcus lactis* was associated with a decreased cellular content of ornithine transcarbamylase, although highly purified preparations of this enzyme did not contain significant amounts of biotin. (Similar situations are not uncommon in the biotin literature.) An indirect role for biotin was indicated, and the authors reported that glycylasparagine in the presence of other peptides replaces the biotin requirement for synthesis of the enzyme. They suggest that the role of biotin in this system apparently is in the production of an active four-carbon unit. Furthermore, the inability to assimilate adequate amounts of exogenous aspartate may account for the diminished synthesis of this enzyme in biotin deficiency. This investigation introduces an interesting concept which envisions the function of biotin in the assimilation of required nutrients.

On the negative side, Bloomfield & Bloch (62), in an investigation of the biosynthesis of 9-dehydro-unsaturated fatty acids by yeast, have noted that biotin has no effect upon the degree of fatty acid desaturation. However, biotin was strikingly involved in the pathway of lipid synthesis from acetate in resting cells. In its absence, fatty acid synthesis was depressed whereas sterol (ergosterol) synthesis was enhanced. Addition of biotin to the biotin-deficient yeast completely reversed the relative rates of these processes. In view of these results, it seems unlikely that biotin plays a role in sterol synthesis from acetate. Mistry & Grillo (63) found no significant alteration in the aspartate and ornithine carbamyl transferase activities of livers from biotin-deficient rats.

Firestone & Koser (64) have investigated the growth-promoting activities

³ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH₂); for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH₂), for triphosphopyridine nucleotide, reduced form (TPNH).

of the biotin analogues, oxybiotin, dethiobiotin, biocytin, norbiotin, homobiotin, and biotinol, for *Candida albicans*. Their results are in general conformity with the previously reported activities of these analogues. Mistry & Dakshinamurti (65) reported on the intracellular distribution of radiobiotin in biotin-deficient rat and chick livers. Ferguson *et al.* (66) have described a microbiological determination of biotin in microgram quantities of tissue. Brown (67) has reviewed the biosynthesis of water-soluble vitamins and their derived coenzymes, which includes biotin, ascorbic acid, nicotinic acid, and the vitamin-B₆ family. In this review, the author fails to mention numerous published papers which clearly demonstrate that oxybiotin, the oxygen analogue of biotin, is not converted into biotin by a variety of microorganisms and the chick. In these organisms, therefore, the biological activity of oxybiotin is an intrinsic property of the molecule and is not caused by the conversion of oxybiotin into biotin.

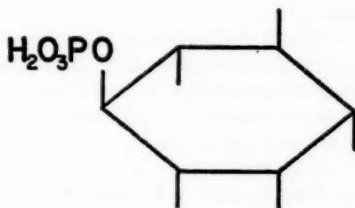


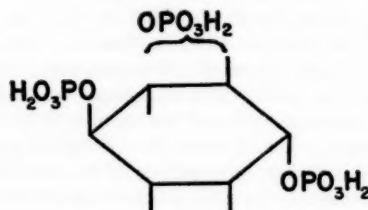
FIG. 1. *Myo*-inositol-4-monophosphate.

INOSITOL

Inositol phosphatides.—The isolation and characterization of the inositol phosphatides represents an active area of investigation. Particular attention is being devoted to the points of attachment of the phosphate moieties to the inositol ring.

Monophosphoinositide, which had until recently been identified only as a component of heart and liver of the animal organism, is a constituent of beef and sheep brain phosphoinositides (68 to 70). Hörhammer *et al.* (68) have observed the monophosphoinositide in both the grey and white matter of beef brain. The diphosphoinositide occurred only in the white matter. Grado & Ballou (69) have reinvestigated the *myo*-inositol phosphates of beef brain phosphoinositide. Base hydrolysis of this phosphoinositide yielded a mixture of one major *myo*-inositol monophosphate, two *myo*-inositol diphosphates, and two *myo*-inositol triphosphates. Each of these components was optically active. The monophosphate was shown to be the 4-phosphate (Fig. 1) and the *myo*-inositol triphosphate mixture to have the structure and absolute configuration shown in Figure 2.

Structures of the diphosphates have not been established. The authors suggest that all of the *myo*-inositol phosphates obtained on base hydrolysis could come from one triphosphoinositide, and they propose a possible mech-

FIG. 2. The *myo*-inositol triphosphates.

anism. Dittmer & Dawson (70) have isolated two triphosphoinositides from ox brain and indicate that they are tightly attached to brain protein. These triphosphoinositides are probably very closely related in structure. Lewin & Wagenknecht (71) have presented further analytical data on an inositol phosphatide from peas.

Hawthorne (72) has studied the point of attachment of the phosphoric acid to the *myo*-inositol ring in liver phosphatidyl inositol. Alkaline hydrolysis of phosphatidyl inositol, glycerylphosphoryl inositol (derived from phosphatidyl inositol) and synthetic glycerol 1-(inositol-2-phosphate) resulted in a mixture of inositol monophosphate and glycerophosphate in proportions which suggested that the inositol of the original liver phosphatidyl inositol is phosphorylated in the 1- or 2-position. Hawthorne *et al.* (73) have isolated inositol-1-phosphate from an enzymatic digest of liver phosphatidyl inositol. This constitutes further evidence for the 1 structure of this inositide. They also carefully purified inositol monophosphate from groundnut phosphatides and they showed it to be optically active. It seems likely that this phosphoinositide also contains the inositol-1-phosphate structure. Ballou & Pizer (74) have established the absolute configuration of the *myo*-inositol-1-phosphate obtained by base hydrolysis of soybean phosphomonoinositide. The absolute configuration of the soybean phosphomonoinositide was assigned as shown in Figure 3. The structure of (+) bornesitol was also confirmed by these authors.

Paulus & Kennedy (75) offered evidence that the enzymatic synthesis

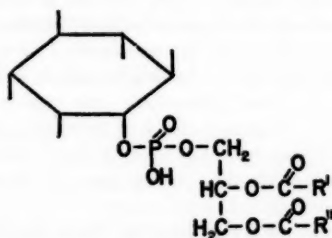
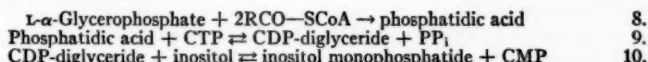


FIG. 3. Soybean phosphomonoinositide.

of inositol monophosphatide in liver preparations proceeds through the following reaction scheme:



Cytidine diphosphate dipalmitin and cytidine diphosphate dilaurin were synthesized by chemical procedures and they acted as intermediates in the above reaction sequence. A previously postulated role of cytidine diphosphate choline in the biosynthesis of inositol monophosphatide has been ruled out in the system studied by Paulus & Kennedy.

Metabolism.—Charalampous (76) reported on the mechanism of action of the purified enzyme from rat kidneys which cleaves inositol to D-glucuronic acid. This enzyme possesses SH groups and one gram atom of iron per mole, both being necessary for its catalytic action. This study, conducted with the aid of inositol-2-C¹⁴ and O¹⁸, indicated that inositol is enzymatically cleaved between carbon atoms 1 and 6, that one atom of oxygen from O₂ is fixed into position 1 of inositol and appears in the carboxyl of glucuronic acid, and that the two hydrogen atoms removed from inositol reduce one atom of oxygen to form water. A possible mechanism for the mode of action of this enzyme has been proposed.

Rajalakshmi *et al.* (77) have studied the influence of inositol upon the esterification of cholesterol. Tissues of the rice moth larvae [*Corcyra cephalonica* (Stainton) strain] and serum of albino rats fed the inositol antagonist, hexachlorocyclohexane, showed a marked increase in cholesterol level, which was manifested mainly in the free cholesterol fraction. Inositol was effective in restoring the ratio of ester to free cholesterol to normal. In a study of the biochemical function of inositol in *Saccharomyces carlsbergensis*, Ghosh *et al.* (78) have observed that inositol deficiency leads to abnormal cell walls containing more glucan than do those from inositol-supplemented cells. These changes were accompanied by the inability of the daughter cells to separate from the parent cells and consequently large cell aggregates were formed. Inositol deficiency did not affect the nucleic acid, protein, mannan, glycogen, pyridine nucleotide, and cytochrome-a, -c, and -a₃ content of the cells or their ability to oxidize glucose, ethanol, and acetate. The concentration of cytochrome-b was greater in cells grown without inositol. Roberts & Yudkin (79) have shown that purified diets that include sodium phytate may cause a conditioned deficiency of magnesium in the rat. This deficiency may be the result of a combination between soluble phytate and dietary magnesium which renders the latter insoluble and unavailable. The study of O'Dell & Savage (80) suggested that phytic acid in soy protein renders dietary zinc unavailable for chicks. Germanier *et al.* (81) have studied the metabolism of inositol in strains of *Neurospora crassa* that are auto- and heterotrophic for this factor. The metabolism was qualitatively the same in both strains and balance studies showed no destruction of inositol. Eagle *et al.* (82) have demonstrated that inositol-independent mouse fibroblasts (strain L-929) can

synthesize inositol from D-glucose and release minute amounts into the medium. Two inositol-dependent cell strains also synthesized significant amounts of inositol.

Methods.—Traisnel & Balatre (83) have described a method of paper electrophoresis for the separation of the inositols and the products of mild oxidation of *meso*-inositol. Perles & Colas (84) presented a microbiological method for the determination of *meso*-inositol in blood. Variations in normal and pathological bloods were determined.

Angyal & Anderson (85) have written a comprehensive review of the cyclitols, and Hawthorne (86) has reviewed the inositol phospholipids.

NICOTINIC ACID

Conversion of tryptophan to nicotinic acid.—Auricchio *et al.* (87) have studied the urinary excretion of the intermediary metabolites involved in the tryptophan-nicotinic acid conversion system in newly born children and sucklings one month old. Their results were consonant with a deficiency of the tryptophan peroxidase activity during the first days of life. Wilson & Henderson (88) have investigated the tryptophan-niacin pathway in the developing chick embryo with particular reference to the role of quinolinic acid as an intermediate. The isotope from tritium-labelled quinolinic and 3-hydroxyanthranilic acids and from tryptophan-7a-C¹⁴ was incorporated into nicotinic and quinolinic acids. Their findings constitute evidence that the tryptophan-niacin relationship established in other species also exists in the chick embryo. Quinolinic acid appears to be involved as a true intermediate in the tryptophan-niacin sequence.

Nutritional aspects.—Gopalan & Srikantia (89) have been impressed by the observation that pellagra is more prevalent in populations whose dietary includes the millet *Sorghum vulgare* (jowar) than in those where rice is the sole staple. The high leucine content of jowar and the observations of Elvehjem and co-workers that dietary supplementation of leucine at a 1 per cent level caused retardation of growth in rats subsisting on low protein diets led these workers to investigate the possible role of amino acid imbalance resulting from a relative excess of leucine in the pathogenesis of pellagra. The oral administration of leucine, as well as the isocaloric replacement of rice by jowar, brought about an increase in the urinary excretion of N-methyl nicotinamide both in healthy subjects and in pellagrins. Exacerbation of clinical symptoms could be reversed by nicotinic acid. The relationship between leucine intake and nicotinic acid metabolism invites further study.

Kodicek and co-workers (90 to 94) substantiate the occurrence of a biologically unavailable form of nicotinic acid in various cereals and the liberation of free nicotinic acid from this bound form by alkaline hydrolysis. Kodicek & Wilson (95) isolated from wheat bran a substance the analysis of which suggests a compound composed of 1 mole nicotinic acid : 1 mole aromatic amine : 6 moles glucose : 2 moles xylose : 1 mole arabinose : about 3 moles substituted cinnamic acids. The authors suggest a tentative struc-

ture for this bound form of nicotinic acid for which they propose the name niacytin.

Metabolic effects.—The clinical use of nicotinic acid to reduce serum cholesterol has been widely accepted; however, the biochemical mechanisms involved in the hypocholesterolemic effects of nicotinic acid have not yet been clarified. Recent studies have been directed toward the elucidation of these mechanisms.

Schade & Saltman (96) have demonstrated a marked inhibition of the rate of cholesterol synthesis by liver slices from rabbits fed nicotinic acid on control or cholesterol-supplemented diets. Since CoA participates in a detoxication mechanism of nicotinic acid via nicotinuric acid as well as in the biosynthesis of cholesterol, the authors suggest that the inhibition of cholesterol synthesis results from a competition between lipid-synthesizing and detoxication systems for a limiting amount of CoA in the liver cell. Cava *et al.* (97) have confirmed that large oral doses of nicotinic acid reduced the hypercholesterolemia and the aortic atheromatosis produced in rabbits by the administration of cholesterol.

Gaylor *et al.* (98) have investigated the effects of high doses of nicotinic acid, nicotinamide, isonicotinic acid, and benzoic acid upon sterol metabolism in the chick and the rat. Although nicotinic acid did not alter the blood cholesterol level of rats fed a hypercholesterolemia-inducing diet, it did depress the blood cholesterol level of chicks fed a cholesterol-containing diet. Nicotinic acid did not alter the total sterol, bile acid, or fatty acid excretion by the rat. Hardy *et al.* (99) reported that liver slices from rats or chicks fed nicotinic acid showed an increased incorporation of acetate into sterols and a decreased incorporation into fatty acids. Incubation of rat liver slices with nicotinic acid also decreased fatty acid synthesis, whereas, in general, it increased the synthesis of sterols. Perry (100) has investigated the effects of nicotinic acid and nicotinamide on the incorporation of acetate into cholesterol, fatty acids, and carbon dioxide by rat liver slices. Nicotinic acid reduced the incorporation of C^{14} -acetate into cholesterol and fatty acids but increased incorporation into CO_2 . Incubation with nicotinamide depressed acetate incorporation into fatty acids but did not affect incorporation of acetate into cholesterol or CO_2 . These results indicate that nicotinic acid diverts a considerable proportion of potential cholesterol and other lipid precursors into an oxidative pathway rather than into the synthesis of cholesterol or fatty acids. Kritchevsky *et al.* (101) reported that the addition of nicotinic acid to normal rat liver mitochondria enhanced the oxidation of the terminal carbon atoms of cholesterol-26- C^{14} . Nicotinamide was without effect. Liver mitochondrial preparations from rats that had been administered nicotinic acid oxidized cholesterol to a greater extent than did similar preparations from control rats. Cholesterol oxidation was increased by the addition of a boiled supernatant fraction from liver preparations of nicotinic acid-fed rats. Similar supernatants from control preparations were without effect. In a study on the relationship between nicotinic acid and cholesterol

metabolism in the rat, Duncan & Best (102) observed that the incorporation of 1 per cent of nicotinic acid in the diet had (a) no significant effect on the incorporation of acetate-1-C¹⁴ into serum and liver cholesterol, (b) no significant effect on serum or liver cholesterol concentrations or on total carcass cholesterol, and (c) no influence upon the absorption of cholesterol-4-C¹⁴ and the disappearance of labelled cholesterol from serum and liver.

Parsons (103) has reviewed his studies on the hypocholesterolemic effect of nicotinic acid in human subjects and has evaluated current hypotheses of its mechanism of action. Miller and co-workers (104) have concluded that the mechanism of action of nicotinic acid in lowering the serum lipid concentrations in man does not appear to be related to depletion of methyl groups or to an effect upon the fecal excretion of bile acids and sterols, and they support the thesis that nicotinuric acid is responsible for the hypolipemic effect of nicotinic acid. Galbraith *et al.* (105) have examined further the effect of large doses of nicotinic acid on the serum cholesterol, total serum lipids, and β -lipoproteins in normal and atherosclerotic humans. Nicotinic acid in doses of three grams daily for two to three weeks caused a prompt reduction in the level of these serum components. Discontinuance of nicotinic acid was followed by a return to pre-treatment values.

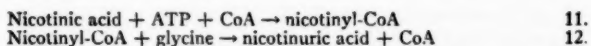
These studies on the relationship between nicotinic acid and lipid metabolism have emphasized the wide differences among species as well as discrepancies of experimental results within a given species. Much further study is required before a complete understanding of the mode of action of nicotinic acid in lipid metabolism is reached.

Wilson & Fostiropoulos (106) have noted that intravenous nicotinic acid will activate plasminogen and, accordingly, will increase the fibrinolytic activity of plasma. The mechanism of action is undetermined. Mascitelli-Coriandoli (107) has reported that the intramuscular injection of nicotinamide into normal rats increased the CoA level of liver. Similar injections were without effect on the CoA level of rat hepatoma.

Metabolism.—Rajagopalan *et al.* (108) have investigated the metabolism of nicotinamide and nicotinic acid in the larvae of the rice moth (*Corcyra cephalonica*) by using radioactive isotopes. Nicotinic and nicotinuric acids were excreted but not N'-methylnicotinamide. Presumably, the nicotinamide deamidase present converts the nicotinamide to nicotinic acid prior to its use in desamido-NAD synthesis. Nicotinamide deamidase from a number of sources is inactivated by metal-chelating agents (109). The enzyme from *Neurospora crassa* could be reactivated by Mn⁺⁺, from *Aspergillus niger* by Mg⁺⁺, and from pigeon liver, chick kidney, and the larvae of the rice moth by Fe⁺⁺.

Conversion of nicotinic acid to both nicotinuric acid and to N'-methylnicotinamide has been observed by Okuda (110) in the rabbit, tortoise, and frog. Further degradation to N'-methyl-6-pyridone-3-carboxamide occurred in the rabbit. An apparent ornithine conjugate of nicotinic acid was excreted by the fowl. Jones & Elliott (111) have determined that ATP and fumarate

are required for nicotinuric acid synthesis in rat kidney slices or homogenates. Since 2,4-dinitrophenol is an inhibitor of this reaction, the authors conclude that reactions involved in oxidative phosphorylation are necessary. The requirement for ATP and oxidative conditions has also been reported elsewhere (108). A more thorough study of this reaction has led to the following formulation of events by Jones (112):



This mechanism is similar to that proposed by Schachter & Taggart (113, 114) for hippuric acid synthesis.

Pyridine nucleotide biosynthesis.—Little additional information to further an understanding of pyridine nucleotide synthesis has accumulated during the past year. Although nicotinic acid is the better precursor for NAD synthesis in human erythrocytes, Dietrich & Friedland (115) have found that both nicotinic acid and nicotinamide serve equally well as precursors of NAD in chicken erythrocytes. They have also demonstrated that the niacin antagonist, 6-aminonicotinamide, is converted by both chicken and human erythrocytes to 6-aminonicotinamide mononucleotide and to 6-amino-NAD. It was not determined whether the synthesis of the latter compound proceeded from 6-aminonicotinamide via NADase or from the 6-aminonicotinamide mononucleotide. In contrast, 6-amino-NAD or 6-aminonicotinic acid-NAD was not formed upon incubation with 6-aminonicotinic acid even though 6-aminonicotinic acid mononucleotide was formed. This inability to be incorporated into a NAD analogue may explain why 6-aminonicotinic acid is a poor niacin antagonist.

Ballio & Russi (116) have detected nicotinic acid, nicotinic acid mononucleotide, desamido-NAD, and NAD in *Fusarium* sp. Since the mononucleotide is also found in *Saccharomyces cerevisiae* and desamido-NAD in *Penicillium chrysogenum*, it is likely that NAD synthesis in fungi proceeds by the same pathways operative in animal tissues (Brown, 67).

Nicotinamide mononucleotide synthesis in human erythrocytes has been observed by Habermann & Habermannová (117) to be initiated at the beginning of the fifth month of life, to reach maximal activity at the age level of ca. six months and thereafter to remain constant. This demonstrates again that human biochemical ontogenesis is not limited to the embryonic and fetal periods of development. In tissues of the fetal guinea pig, NAD and NADH₂ levels were found by Nemeth & Dickerman (118) to remain unchanged throughout the gestation period. NADPH₂ was low in the fetal liver but rose to adult values shortly after birth. NADase activity increased at the end of gestation and reached adult values shortly before birth.

Methods.—The spectroscopic determination of nicotinamide and of nicotinuric acid after removal of interfering materials by chromatography has been described by Jones (119). Nicotinuric acid in the range of zero to 100μg. and nicotinamide in quantities from zero to 40μg. could be determined.

VITAMIN B₆

Vitamin-B₆ enzymes.—Continuing their excellent studies on the purified glutamic-aspartic transaminase from pig heart, Jenkins & Sizer (120) have prepared the pyridoxal and the pyridoxamine forms of the enzyme. The stoichiometry of the reaction between the disappearance of the pyridoxal form of the enzyme and the appearance of α -ketoglutarate and enzyme-bound pyridoxamine phosphate following the addition of glutamate provides evidence that transamination is a reaction between enzyme and substrate; i.e., the reaction proceeds consecutively and does not involve the simultaneous participation of both substrates. The decrease in absorbancy at 362 m μ following the addition of glutamate is a resultant of the formation of the enzyme-substrate complex(es) and the pyridoxamine enzyme. Although the reaction between glutamate, α -ketoglutarate, and enzyme was investigated spectroscopically in some detail, it was not possible to identify unequivocally the complexes so formed. The possibility of metal involvement in enzyme-catalyzed transaminations has been raised again by Patwardhan (121). A partially purified glutamic-aspartic transaminase from *Dolichos lablab* was inactivated by dialysis or by the addition of chelating agents; partial restoration of activity could be obtained by the addition of Fe⁺⁺. The stimulation by Fe⁺⁺ was not the result of a non-enzymic reaction contributing to the rate. Maximal reactivation after dialysis also required the addition of pyridoxal phosphate. Supplementary evidence for the participation of iron in this reaction was presented in the form of an increase in the iron concentration per mg. protein through a tenfold purification of the enzyme and an approximation to constancy of the specific activity to iron concentration ratio. It would be premature, however, to conclude that the transaminase is an iron-containing enzyme. An investigation of transamination reactions in *Neurospora crassa* by Seecof & Wagner (122) resulted in a fifteen- to thirtyfold purified enzyme preparation as determined with the donor substrates, phenylalanine and α -keto- β -methylvalerate. Only trace activity toward alanine, aspartate, and ornithine was observed, but the preparation readily transaminated between phenylalanine and various keto acids and between phenylpyruvate and other donor amino acids. The authors (123) have also examined various parameters that affect the rate of transamination and have concluded that the kinetic results (with phenylalanine as the donor amino acid) are compatible with the viewpoint that a single enzyme mediates the conversion of α -keto- β -methylvalerate and α -keto-isovalerate to isoleucine and valine, respectively. β -Fluoro-oxaloacetate has been shown by Kun *et al.* (124) to inhibit competitively the glutamic-aspartic transaminase of heart mitochondria. Reaction rates were determined by spectral measurement of the enol-borate complex of the formed oxaloacetate. The inhibitor will undergo a slow transamination with aspartate to yield β -fluoro-aspartate which is presumed to undergo subsequent dehydrofluorination and deamination to oxaloacetate.

Ono & Hagen (125) have demonstrated that pyridoxal phosphate subserves the function of a coenzyme in the histidine decarboxylase from mouse

mastocytoma. The activity was not diminished by dialysis for 24 hr., but after 60 hr. the addition of pyridoxal phosphate was necessary for the restoration of activity.

A glutamic decarboxylase from *Escherichia coli* (strain 26) has been extensively purified by Shukuya & Schwert (126). Ultracentrifugal and electrophoretic analysis gave an indicated purity of 90 per cent with a molecular weight of 300,000. The enzymatic decarboxylation reaction, with a pH optimum of 3.8, was stimulated by the addition of pyridoxal phosphate, but its effect on the apoenzyme could not be determined since the holoenzyme could not be resolved. However, pyridoxal phosphate could be dissociated from the enzyme in 0.1 N sodium hydroxide, and spectrophotometric evaluation of its liberation permitted a value of two moles pyridoxal phosphate per mole of enzyme to be assigned (127). The spectral properties of the enzyme were markedly dependent upon pH; a shift from neutrality to acidic solution produced a yellow enzyme with an increase in the absorbancy at 415 to 420 m μ and a decrease in the absorbancy at 340 m μ . From an analysis of the absorbancy change in going from the unprotonated to the protonated form of the enzyme, a structure with four dissociable groups having an apparent dissociation constant corresponding to a pK' of 5.61 was implicated. Observation of the changes in the absorption spectra as a function of time following glutamate addition indicates that the active form of the enzyme is the species which absorbs at 415 m μ , i.e., the protonated form. Upon addition of glutamate at pH 4.6, the absorbancy at 415 m μ was diminished with a concomitant increase in absorbancy at 330 m μ . These changes were reversible with time. Finer details of this interaction were obtained by the more sensitive methods of fluorescence-emission spectroscopy. Interaction of the enzyme with glutamate at pH 4.6 produced maximum quenching of the 490 m μ emission (activation at 420 m μ) instantaneously, and after a measurable lag period of several seconds maximum augmentation of the 380 m μ emission (activation at 335 m μ) occurred. Since the product of the reaction, α -aminobutyrate, does not quench the 490 m μ emission, Shukuya & Schwert consider it probable that both this effect and the diminution of the 415 m μ absorption is caused by disruption of the hydrogen-bonded ring chelate between apoenzyme and coenzyme by glutamate with resultant Schiff base formation. The increase in 380 m μ fluorescent emission is likewise not attributable to the accumulation of α -aminobutyrate but may result from the accumulation and subsequent disappearance (after substrate exhaustion) of the free aldehyde form of enzyme-bound pyridoxal phosphate.

The purified glutamic acid decarboxylase loses activity more rapidly at 0° than at 25°C. (128). The loss could be prevented in part in solutions of high ionic strength, high dielectric constant, or by the addition of pyridoxal phosphate or bovine serum albumin. The process has been interpreted as a dissociation in which the equilibrium constant of the reaction favors formation of the apoenzyme with resultant change in protein conformation.

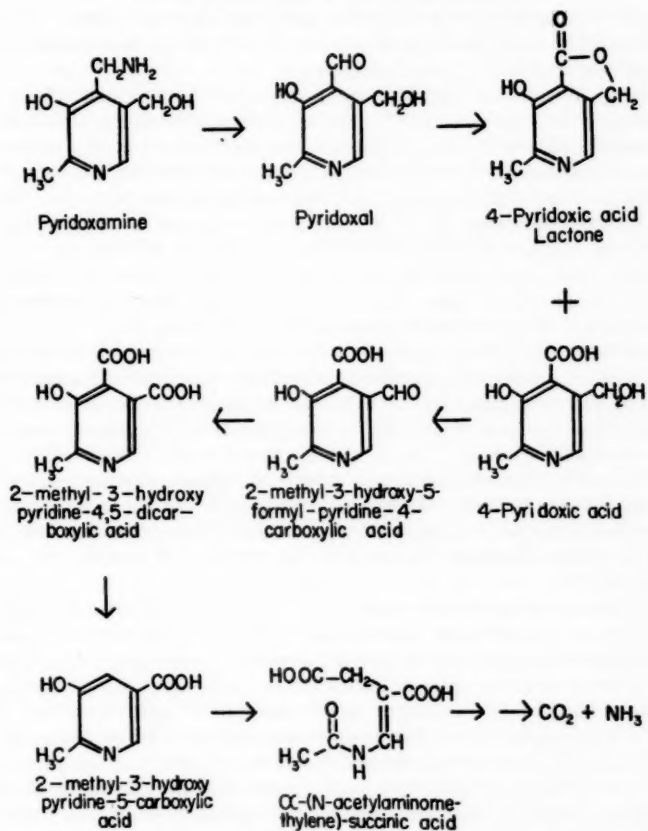
Cowgill (129) has purified the phosphorylase-*a* from lobster muscle to a state of apparent homogeneity and found that it contained one mole pyri-

doxal phosphate per 100,000 gm. protein. Adenosine monophosphate was not required for activity. A phosphorylase-*b* was also isolated from the same source, and, although it required AMP for activity, it differed from the rabbit muscle enzyme in that its molecular size approximated that of the phosphorylase-*a*. Evidence for a third enzyme, designated phosphorylase-*c*, was also presented. It could be readily converted into phosphorylase-*b* and had a much higher requirement of AMP for activity. The instability of this component prevented purification. Cowgill (130) has partially purified a phosphorylase phosphatase and a phosphorylase kinase from lobster muscle which were capable of converting phosphorylase-*a* to -*b* and of phosphorylase-*b* to -*a*, respectively.

The mechanism of inhibition of B_6 -requiring enzymes by isonicotinic acid hydrazide has been considered to involve the formation of a hydrazone by interaction with the aldehyde moiety of pyridoxal phosphate. However, this mechanism of the inhibition is in apparent contradiction to the observations of Bonavita & Scardi (131) and of Gonnard & Nguyen-Philippou (132), who reported that the isonicotinyl hydrazone of pyridoxal phosphate activated the glutamic-aspartic apotransaminase. The hydrazone has also been demonstrated to be an activator for dihydroxyphenylalanine-decarboxylase (133) and for kynureninase (134). Both groups of workers discount the possibility of the hydrazone releasing pyridoxal phosphate, and, in view of these results, the role of the aldehyde group in such enzymic reactions requires further investigation. Heilbroun (135) has reported that, whereas hydrazines, hydrazides, and hydrazones were inhibitors of diamine oxidase, none of these compounds affected the activity of brain glutamic acid decarboxylase. She concludes that the reaction between hydrazines or hydrazides and pyridoxal phosphate cannot be the sole reason for the inhibition of a B_6 -requiring enzyme.

Alioto (136) studied the isonicotinic acid hydrazide inhibition of the glutamic-alanine transaminase in various tissues, some of which convert isonicotinic acid hydrazide to isonicotinic acid and hydrazine with subsequent decomposition of the latter to ammonia. The effective inhibitor was found to be isonicotinic acid hydrazide and not its breakdown products. Isonicotinic acid hydrazide has been exploited by Sidorova (137) to obtain a greater impairment of transamination in B_6 -deficient mice. The glutamic-alanine transaminase was almost totally suppressed, whereas the glutamic-aspartic enzyme was only 40 to 50 per cent inhibited. This latter degree of inhibition correlated well with the concomitant 50 to 60 per cent inhibition *in vivo* of urea synthesis since one of the urea nitrogens arises from aspartate in the transamination between glutamate and oxaloacetate.

Cycloserine, cyclothreonine, the ethyl ester of β -amino oxalalanine and β -amino oxalalanine have been tested as inhibitors of the glutamic-alanine transaminase in rat liver by Vyshepan *et al.* (138). Cycloserine and the ethyl ester of β -amino oxalalanine (capable of yielding cycloserine at the pH of the enzyme reaction mixture) produced 50 to 100 per cent inhibition in concentrations of 10^{-3} to 10^{-4} M. The other two compounds were much less effective.

FIG. 4. Pyridoxamine oxidation by *Pseudomonas* sp. MA.

Addition of pyridoxal phosphate did not restore activity, and it was concluded that an addition compound with the enzyme-bound coenzyme had been formed.

Metabolism.—*Pseudomonas* sp. IA degrades pyridoxine through isopyridoxal, 5-pyridoxic acid, pyruconic acid, and hence to smaller fragments (139, 140). More recently, Burg, Rodwell & Snell (141) have isolated by chromatography on a Dowex 1-formate column the products of pyridoxamine metabolism by another strain, *Pseudomonas* sp. MA. The reaction pathway in Figure 4 was constructed from the structures of the isolated products; the sequence is inferred from the oxidation states involved and the order of their appearance in the culture filtrate. The initial steps are similar to those oc-

curing in mammals (to 4-pyridoxic acid), and, although none of the products from the oxidation of pyridoxine by the IA culture was observed, some of the reaction steps proceed analogously.

A pyridoxine-oxidizing enzyme from rabbit liver which will convert pyridoxine to 4-pyridoxic acid in the presence of aldehyde oxidase has been purified fiftyfold by Morino *et al.* (142). The purified enzyme did not produce detectable pyridoxal from pyridoxine, and 4-pyridoxic acid was formed only in the presence of the pyridoxine oxidase and the aldehyde oxidase. The authors therefore assume that the latter enzyme acts as a trapping agent to drive the reaction from the equilibrium-flavored pyridoxine to pyridoxal. Morisue and co-workers (143) have also purified a pyridoxine phosphate oxidase from rabbit liver. The enzyme contains either flavin-adenine dinucleotide or flavin mononucleotide as the prosthetic group.

Methods.—B₆-free tyrosine decarboxylase has been used by Wachstein *et al.* (144) to determine pyridoxal phosphate in plasma and leukocytes of normal and pregnant subjects following B₆-load tests. Bonavita (145) has studied the reaction of cyanide with pyridoxal and pyridoxal phosphate. Characteristic changes in both the absorption and fluorescence-emission spectra are noted with both compounds. With fluorescent emission as the detector, 1×10^{-5} to $5 \times 10^{-8} M$ pyridoxal phosphate can be determined. Pyridoxal interferes with the assay, but, provided its concentration is approximately equal to or lower than that of the pyridoxal phosphate, the effect is negligible.

Evaluation of pyridoxine nutriture.—Methods for the precise evaluation of the status of pyridoxine nutriture are under investigation. Assessment of borderline deficiency states in man will depend in large measure upon the development of such procedures. Babcock *et al.* (146) have studied the response of the serum glutamic-oxalacetic transaminase to the administration of pyridoxine as a measure of pyridoxine nutriture in rats and in man. This criterion was less sensitive than the xanthurenic acid excretion test. In both young and adult rats, Brin *et al.* (147) have demonstrated that the alanine transaminase of plasma was more markedly affected by a pyridoxine deficiency than the aspartic enzyme. Incubation *in vitro* with pyridoxal phosphate partially restored enzymatic activity. Cheslock & McCully (148) have studied eight human subjects maintained for 52 days on a diet low in pyridoxine. The blood content of pyridoxine dropped to zero within four weeks, and the xanthurenic acid excretion after a test dose of tryptophan exceeded 30 mg. per day in five of the subjects. Wachstein *et al.* (144) have suggested that the level of pyridoxal phosphate in plasma and leukocytes, particularly after the administration of pyridoxine, may prove useful in assessing the state of pyridoxine nutrition. Benhamou & Amouch (149) have described a paper electrophoresis technique for the urinary determination of pyridoxine, pyridoxamine, pyridoxal, pyridoxamine phosphate, and pyridoxal phosphate as a screening test for pyridoxine deficiency. The existence of a mild state of pyridoxine deficiency among the aged has been indicated by the experiments of Ranke *et al.* (150). The authors utilized serum transaminase levels

and xanthurenic acid excretion after a tryptophan load as indices of pyridoxine nutrition. Further efforts must be directed toward the development of more sensitive procedures for the assessment of pyridoxine nutriture and their application to experimental subjects, particularly man.

Metabolic effects.—Experiments by Parkes (151) have emphasized the important influence of diet upon the homograft rejection phenomenon. He demonstrated that the treatment of recipient mice with deoxypyridoxine increased markedly the survival rate of intra- as well as interstrain ovarian homografts. Of particular note were the striking effects obtained with heterografts of rat ovaries into deoxypyridoxine-treated mice. These results are comparable to those of Axelrod *et al.* (152) and Fisher *et al.* (153) obtained with rat skin homografts.

The role of pyridoxine in the cellular transport mechanism of amino acids continues to be a subject of active interest. Jacobs *et al.* (154) have demonstrated the inhibition of intestinal absorption of L-methionine by deoxypyridoxine and 2,4-dinitrophenol in the rat. Rats maintained on a pyridoxine-deficient diet also exhibited a depressed absorptive ability. The inhibition of intestinal absorption by deoxypyridoxine was alleviated by pyridoxine. 2,4-Dinitrophenol-induced inhibition was not affected by pyridoxine but could be alleviated with pyridoxal phosphate, which is explicable on the basis that 2,4-dinitrophenol blocks the phosphorylation reaction involved in the synthesis of pyridoxal phosphate which is essential for the absorptive process. Akedo and co-workers (155) have shown that the intestinal absorption of L-methionine, L-histidine, and L-lysine is decreased markedly in pyridoxine-deficient rats. Normal activity is restored by pyridoxine.

A recent series of papers deals with the effects of a pyridoxine deficiency in pregnancy. Brown (156) has noted that the adverse effect of a pyridoxine deficiency on fertility and reproductive performance of rats was accentuated when hydrogenated shortening rather than corn oil was the source of dietary fat. In studies on changes that might be related to toxemia, Brown & Pike (157, 158) have investigated the effect of a pyridoxine deficiency upon the parameters of blood pressure, thiocyanate space, blood volume, and serum protein in the pregnant and non-pregnant rat. Hypertension was not observed in pyridoxine-deficient pregnant or non-pregnant animals. When calculated on the basis of body weight, thiocyanate space increased in the deficient rats. This effect was not intensified during pregnancy.

Emerson *et al.* (159) have summarized their studies on the effects of graded levels of butterfat and safflower oil in rhesus monkeys maintained for successive periods on various levels of pyridoxine. Elevation of plasma lipids and their fractions appear to be dependent upon the intake of pyridoxine and the level and type of fat in the diet. Goswami & Sadhu (160) have reported a lowered serum level of tetraenoic acids in hypercholesterolemic pyridoxine-deficient rats.

Andrus *et al.* (161) have demonstrated the formation of renal papillary apical concretions composed of calcium oxalate monohydrate in pyridoxine-deficient white rats receiving dietary supplements of glycine. Acidification of

the urine enhanced the development of the concretions. These calculi produced in the rat resembled closely the oxalate calculi seen in man. The biochemical mechanisms of the increased oxalate excretion in pyridoxine deficiency and the relationship of these findings to man remain to be determined. Richert and co-workers (162) have investigated the rate of heme synthesis in reticulocyte-rich blood induced by the administration of phenylhydrazine. Heme synthesis was decreased to about one-third of the control level in erythrocytes from pyridoxine-deficient ducks. Addition of pyridoxal-5'-phosphate to the deficient cells restored heme synthesis to control values.

Berdjis *et al.* (163) have noted that monkeys maintained on diets deficient or suboptimal in pyridoxine for 16 months or longer exhibited a marked increase in the incidence of dental caries. It appears that pyridoxine deficiency exerts its various deleterious effects on the permanent teeth during the course of their development. Atrophic glossitis and acute and chronic gingivitis occurred in the deficient monkeys. Mihich *et al.* (164) have described the effects of a dietary depletion of pyridoxine on the growth of a spectrum of tumors. Growth of six mouse tumors and of one rat tumor was impaired in this deficiency state. Williams & Wiegand (165) support the thesis that the seizures produced by isonicotinic acid hydrazide and three other convulsant hydrazides are attributable to the production of an acute pyridoxine-deficiency state in the dog. Eisenstein (166) has shown that the glucocorticoid action of cortisol, as well as the effect of the hormone upon liver glutamic-pyruvic transaminase, was diminished in pyridoxine-deficient rats. These findings emphasize the importance of the nutritional state in defining hormonal activity and support the concept that glucocorticoid activity may depend upon increased levels of liver glutamic-pyruvic transaminase.

Miscellaneous.—Knapp (167) has described a genetically dependent abnormality in tryptophan metabolism which was evidenced by an excessive excretion of tryptophan metabolites following a tryptophan load test. This metabolic derangement was reversed by the administration of pyridoxine. All the evidence argued for a higher familial requirement for pyridoxine to maintain a normal state of tryptophan metabolism. Ranke and co-workers (168) have studied the interrelationship between vitamins B₆ and B₁₂ in the rat. Thus, there is further evidence for vitamin interrelationships, and the data emphasize the possible effects of a single vitamin-deficiency state upon the metabolism of other vitamins. Bernhart *et al.* (169) provide evidence for a sulfur-containing compound with some vitamin-B₆ activity in heat-sterilized milk. This product was formed by reacting pyridoxal with cysteine at pH 7.0 and 100°C. Subsequently, Wendt & Bernhart (170) gave the structure of this compound as shown in Figure 5.

Thyroxine can inhibit, both *in vivo* and *in vitro*, a number of enzymatic systems that require pyridoxal-5'-phosphate as a coenzyme. Mascitelli-Coriandoli & Boldrini (171) have demonstrated a decrease in the liver and heart contents of pyridoxine and pyridoxal phosphate in thyroxinized rats. The administration of pyridoxine increased the pyridoxine concentrations of both tissues without effecting a significant change in pyridoxal phosphate

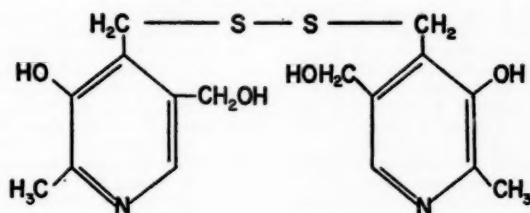


FIG. 5. Bis-4-pyridoxyl disulfide.

concentrations. Conversely, no changes in pyridoxine levels were induced by adenosine triphosphate administration, whereas the pyridoxal phosphate levels were increased. Labouesse *et al.* (172) have also noted a decrease in the liver pyridoxal phosphate content after injections of thyroxine and an increase following thyroidectomy. Ashwood-Smith & Smith (173) have reported that the reduced glutathione content of brain cortex, cerebellum, and medulla is diminished in pyridoxine-deficient rats.

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WATER-SOLUBLE VITAMINS, PART II^{1,2}

(FOLIC ACID, RIBOFLAVIN, THIAMINE, VITAMIN B₁₂)

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The biogenesis of the vitamins and their derivatives, as well as certain aspects of their mode of action, has been emphasized in this review.

RIBOFLAVIN AND FOLIC ACID

INTERRELATIONSHIP OF THE BIOGENESIS OF FLAVINS, PTERIDINES, AND PURINES

A number of studies have recently converged to suggest a common metabolic origin of certain molecular portions of the purines, pteridines, and flavins. Thus, the label from simple precursor substances is incorporated into analogous positions in structurally related moieties of these substances; the biosynthesis of flavin is enhanced in the presence of purines and pteridines; purines can be chemically converted to pteridines under very mild temperature and pH conditions (1, 2); and a major part of the molecule of some purines is incorporated intact into the analogous positions of riboflavin and certain pteridines. Two ribitylpteridines structurally similar to riboflavin have been isolated from riboflavin-producing organisms, and one of these is enzymically converted to riboflavin. Evidence bearing on the metabolic interrelationship of these substances is summarized in the subsequent discussion.

The incorporation of labeled organic compounds of small molecular weight.—A remarkable similarity in the pattern of labeling in analogous portions of the purine and flavin molecules was demonstrated when cells of *Ashbya gossypii* and *Eremothecium ashbyii* were grown with radioactive formate, carbon dioxide, glycine, and glycine-N¹⁵ (3 to 5). This similarity in chemical structure and mode of biosynthesis has been extended to the pteridines by the studies of Weygand & Waldschmidt (6) on the incorporation of radioactive formate and glycine into leucopterin by the butterfly *Pieris brassicae* and on the distribution of label in certain ribitylpteridines (see below). Brenner-Holzach & Leuthardt (7) also reported incorporation of label from glycine-2-C¹⁴ and formate-C¹⁴ into uric acid, drosoppterin, sepiapterin, and isoxanthoppterin by

¹ The survey of the literature pertaining to this review was concluded in October, 1960.

² The following abbreviations are used: CoA for coenzyme A; FAD for flavin-adenine dinucleotide; NAD for nicotinamide-adenine dinucleotide; NADH₂ for nicotinamide-adenine dinucleotide, reduced form; NADP for nicotinamide-adenine dinucleotide phosphate; NADPH₂ for nicotinamide-adenine dinucleotide phosphate, reduced form.

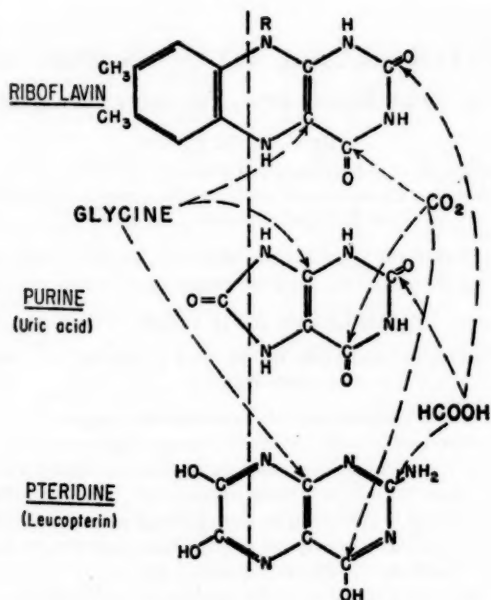
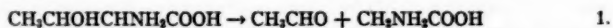


FIG. 1. Incorporation of various labeled substances into riboflavin, purines, and pteridines.

Drosophila melanogaster, but the position of the label was not reported. These relationships have been summarized in Figure 1.

The labeling depicted (Fig. 1) has received further support in the case of riboflavin from the work of Goodwin & Jones (8). Label from serine-3-C¹⁴ was fixed primarily in carbon 2 of riboflavin by *E. ashbyii*, with a trace in carbons 4a + 9a; with uniformly C¹⁴-labeled serine, radioactivity was found exclusively in carbon 2 and carbons 4a + 9a of the riboflavin molecule. "Active" formate from the beta-carbon of serine and of glycine from the alpha-carbon and carboxyl groups is probably formed here too by known metabolic pathways (9); these observations with *E. ashbyii* are thus in agreement with those with *A. gossypii* (10). Goodwin & Horton (11) have shown that the label from uniformly C¹⁴-labeled threonine was fixed only into positions C_{4a+9a} of riboflavin by *E. ashbyii*, i.e., into the carbons known to be contributed by glycine (10). In this case, glycine is probably formed from threonine by way of threonine aldolase:



The latter results make untenable the previous explanation for threonine

stimulation of flavin production in *E. ashbyii* (12), which proposed that two molecules of threonine might condense to the *o*-xylene moiety of riboflavin.

Purines and flavins.—Stimulation of riboflavin formation by the addition of purines to the growth medium has been observed with such flaviogenic organisms as *Eremothecium ashbyii* (13 to 15) and *Candida flareri* (18). The corresponding purine ribosides are not superior to the free bases (12, 14) and no incorporation into the ribityl group of riboflavin from the riboside group of uniformly C¹⁴-labeled guanosine was observed (72).

However, it is frequently misleading to interpret the effect on fermentation yield of added substances in terms of a precursor-product relationship. For example, Yaw (16) and Minoura (17) could not obtain stimulation of flavin synthesis by selected purine bases or nucleic acid hydrolyzates in *E. ashbyii*; intact nucleic acid was effective under Minoura's conditions but inferior to peptone. Furthermore, Brown *et al.* (15) observed that such substances as 2-methyladenine and 2-methylhypoxanthine have an excellent flaviogenic effect with *E. ashbyii*; these compounds, unless the organism is capable of demethylation, should not serve as precursors in light of the results with labeled purines (see below). Alternatively, the 2-methyl purines inhibit some phase of purine synthesis, and precursors which accumulate may then be diverted to flavin synthesis. This has been suggested for the flaviogenic effect of 4-aminopterlin (15).

Many compounds other than purines affect riboflavin formation in a variety of organisms, and at present it is difficult to interpret their action in terms of mechanisms of flavin biosynthesis. For example, stimulation of flavin production by urea in *Candida flareri* (18) is not accompanied by incorporation of radioactivity from urea-C¹⁴ into riboflavin (19).

However, definitive evidence for a structural precursor-product relationship between purines and flavins was presented by McNutt (14), who showed a pronounced incorporation of label from uniformly C¹⁴-labeled adenine into riboflavin with growing cells of *E. ashbyii* but little incorporation from adenine-8-C¹⁴. The radioactivity in riboflavin was located almost exclusively in rings B and C with approximately equal distribution in the constituent carbons of these rings (20). This suggests that the portion of the purine ring system exclusive of carbon 8 can be utilized directly for rings B and C without prior degradation to smaller precursor compounds. By degradation of the riboflavin formed by *E. ashbyii* in the presence of uniformly N¹⁵-labeled adenine, McNutt further demonstrated that the nitrogen atoms from the pyrimidine and imidazole moiety of the purine were also transferred intact to the vitamin (21). Pyrimidines per se are probably not precursors of rings B and C of riboflavin, since the radioactivity of added orotic-6-C¹⁴ acid was not recovered in riboflavin synthesized by *Ashbya gossypii* (4, 22) and other organisms (23).

The direct convertibility of the major part of the purine skeleton to rings B and C raises the question of whether a purine is an obligatory intermediate in the biosynthesis of the vitamin, i.e.,

The isolation, synthesis, and metabolic properties of 2,4-dihydroxy-8-ribitylpteridines.—Masuda (29) isolated a number of substances from the mycelium of *Eremothecium ashbyii*. Aside from riboflavin, considerable amounts of FAD were encountered (30), and under suitable fermentation conditions commercial preparation of this coenzyme by this route appears feasible (e.g., 31 to 34). In addition to flavins, however, a number of substances showing strong fluorescence under ultraviolet light were detected; some were crystallized; one was identified as L-3-hydroxykynurenine (35). Two of the substances, exhibiting violet (compound V) and green (compound G) fluorescence, were examined in considerable detail by Masuda and collaborators (36 to 39).

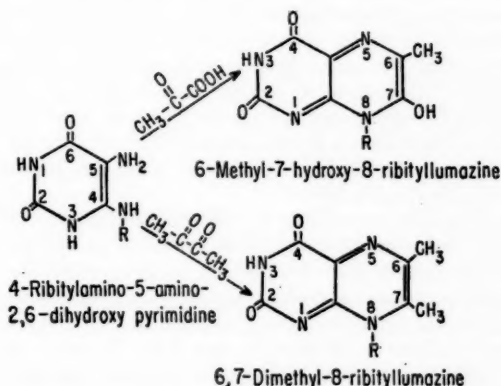


FIG. 2. Chemical synthesis of 6,7-dimethyl-8-ribityllumazine and 6-methyl-7-hydroxy-8-ribityllumazine.

Maley & Plaut (40, 41) found a pronounced incorporation of radioactivity into two substances (different from flavins) upon the addition of formate- C^{14} to growing cultures of *Ashbya gossypii*; one fluoresced blue and the other green. The green material was isolated in pure form from the mycelium of this organism. On the basis of spectrophotometric analyses and certain chemical degradations, Masuda (37, 42) assigned the structure of 6,7-dimethyl-8-ribityllumazine (6,7-dimethyl-8-ribitylpteridine-2,4-dione) to compound G. This substance was prepared by chemical synthesis (Fig. 2) by Maley & Plaut (40, 41) and was identical with the green fluorescent material isolated from *A. gossypii*; compound G possesses the same properties as a synthetic material of this structure (43). 6,7-Dimethyl-8-ribityllumazine has also been isolated from *Clostridium acetobutylicum* (44); other publications on its chemical synthesis have appeared (45 to 47).

The possibility that 6,7-dimethyl-8-ribityllumazine is a precursor of ribo-

flavin is suggested by the striking similarity of its chemical configuration to that of the vitamin, the fact that chemical condensation of the lumazine and diacetyl led to the formation of riboflavin (38, 48), and the observation that *A. gossypii* incorporates radioactivity from formate- C^{14} into analogous positions (carbon 2) of the ring structures of the two compounds (41). Furthermore, studies on the rates of incorporation by *A. gossypii* of a number of labeled substances (formate, glycine, adenine) revealed that the molar specific radioactivity of 6,7-dimethyl-8-ribityllumazine was always larger than that of riboflavin in the early periods of incubation. This makes it unlikely that the lumazine derivative arises as a degradation product of riboflavin in the organism and suggests that it is an intermediate in the biogenesis of the vitamin (40, 41). This conclusion has been confirmed by experiments with extracts from a number of microorganisms in which an enhanced formation of riboflavin was found upon the addition of 6,7-dimethyl-8-ribityllumazine (49 to 53).³ The molar specific radioactivity of riboflavin formed upon incubation of extracts of *A. gossypii* with radioactive 6,7-dimethyl-8-ribityllumazine-2- C^{14} is of the same magnitude as that of the added lumazine derivative. Thus, the lumazine is utilized for formation of the flavin and does not merely facilitate the catalytic conversion of another component in the crude extract to the vitamin (53).

Extracts capable of converting 6,7-dimethyl-8-ribityllumazine to riboflavin were obtained from microorganisms known to grow in media without added riboflavin. *Lactobacillus casei*, which requires riboflavin for growth, does not appear to possess conversion activity (40, 52, 54). Thus, the distribution of the conversion activity is consistent with the role proposed for lumazine as an intermediate in riboflavin biosynthesis. However, the report of Katagiri *et al.* (54) that extracts from beef liver are also capable of effecting the conversion may not be in accord with such a correlation.

Substrate specificity may be another important factor in assessing the significance of the conversion reaction in the biogenesis of the vitamin. This reaction has been studied with compounds possessing different substituents on the lumazine ring. Replacement of the methyl group on carbon 7 by a

³ Korte and co-workers (23, 52) suggest that 6,7-dimethyl-8-ribityllumazine is not an intermediate in riboflavin synthesis, since intact cells were unable to convert this compound to flavin, although extracts of certain microorganisms do convert this compound to flavin. However, these results are not necessarily conclusive since many cases are known in which added substance is not (or poorly) utilized by an intact cell but has, nevertheless, been proven to be an intermediate of a metabolic pathway within the cell (cryptic mutants). They also demonstrated that incorporation of radioactivity from guanine-5- C^{14} into 6,7-dimethyl-8-ribityllumazine and riboflavin did occur with intact cells but not with extracts from the same organisms. These apparently contradictory results arise from an incomplete recovery in the extract of a step required for transformation of purine to the ribitylpteridine, or the more successful competition by other reactions for an essential intermediate upon disruption of the cell organization.

hydroxyl group (6-methyl-7-hydroxy-8-ribityllumazine; see below) (49, 56) and of the ribityl by either a hydrogen (6,7-dimethylumazine) or a methyl group (6,7,8-trimethylumazine) (51, 55) did not lead to the formation of the corresponding flavin derivative. However, Katagiri *et al.* (57) reported that intact cells of *Clostridium acetobutylicum* and enzyme preparations from *Aerobacter aerogenes* and *Eremothecium ashbyii* did form 6,7-dimethyl-9-(2'-hydroxyethyl)isoalloxazine in the presence of 6,7-dimethyl-8-(2'-hydroxyethyl)umazine. In contrast, no such conversion of the hydroxyethyl compound could be noted in studies of this compound with preparations from *Escherichia coli* (ATCC 9637) and *Ashbya gossypii* (55). Indeed, the results of Johnson & Plaut (58) with purified enzyme from *A. gossypii* emphasize the specificity of the reaction for the substituent on position 8, since, in con-

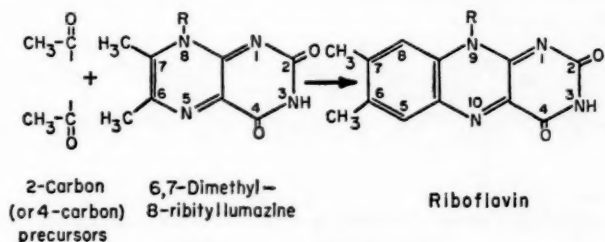


FIG. 3. Schematic representation of conversion of 6,7-dimethyl-8-ribityllumazine to riboflavin.

trast to 6,7-dimethyl-8-D-ribityllumazine, neither substrate disappearance nor flavin formation could be detected when the following substances were tested in their system: 6,7-dimethyl-8-D-arabityllumazine, 6,7-dimethyl-8-L-arabityllumazine, 6,7-dimethyl-8-D-lyxitullumazine, 6,7-dimethyl-8-D-xylityllumazine, or 6,7-dimethyl-8-D-galactityllumazine. Though not converted to flavins, some of these compounds do exhibit an affinity for the enzyme system, since 6,7-dimethyl-8-D-xylityllumazine is a competitive inhibitor of the conversion of 6,7-dimethyl-8-D-ribityllumazine to riboflavin.

The formation of riboflavin from 6,7-dimethyl-8-ribityllumazine requires the addition of four carbon atoms for the completion of the *o*-xylene ring of the vitamin (Fig. 3). Masuda (38, 48) presented evidence that 6,7-dimethyl-8-ribityllumazine could be chemically converted to riboflavin in the presence of acetoin or 2,3-butanedione, and he has suggested that this reaction occurs biologically. On the other hand, Cresswell & Wood (45) obtained riboflavin (Fig. 4, IV) by the chemical condensation of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (I) with a dimer of 2,3-butanedione (II) by way of an intermediary pteridine (III) (Fig. 4); this is in agreement with Birch & Moye (59, 60), who found no lumiflavin when 6,7,8-trimethylumazine was treated with 2,3-butanedione (60). 2,3-Butanedione does not appear to fulfil the

biological role proposed by Masuda (38, 48), since Katagiri *et al.* (50) (see also 53, 62) found it did not stimulate the conversion of the ribityllumazine to riboflavin with various preparations from *Clostridium acetobutylicum* and *Escherichia coli neapolitanus*. However, they (51) reported beneficial effects of acetaldehyde, acetate, and pyruvate on this reaction; it should be noted that the conversion of 6,7-dimethyl-8-ribityllumazine proceeded at 50 to 70 per cent of the rate when these substances were absent [cf. Table II in (51)]. Evidence was presented that these compounds are converted to an active acetyl compound before addition to the ribityllumazine to form flavin. Using enzyme preparations from *E. coli neapolitanus* and *Cl. acetobutylicum*, Katagiri *et al.* (54) reported that the transformation of the ribityllumazine to riboflavin in the presence of acetate required ATP, CoA, and NADH₂ for full activity. The combination of acetate and ATP could be replaced by acetyl

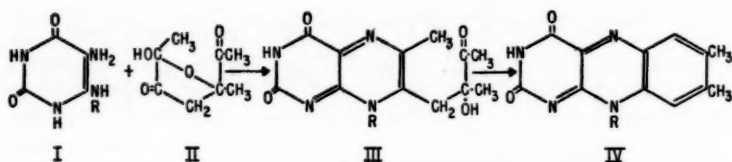


FIG. 4. Chemical formation of riboflavin from a diaminouracil and a dimer of 2,3-butanedione:
R = H, CH₃, or ribityl

phosphate; hence, it would seem that acetyl CoA is the actual precursor of carbons 6+7 and the methyl groups of riboflavin. These results appear to be consistent with the positioning of label in the *o*-xylene moiety of riboflavin obtained with labeled acetate and glucose in growing cultures of *A. gossypii* (61). However, since in the latter experiments label in ring A from added acetate-2-C¹⁴ was more randomized than that from glucose-1-C¹⁴ or glucose-6-C¹⁴, it was considered unlikely (61) that two-carbon fragments from the carbon 1+2 and 5+6 portion of glucose pass through an acetate pool prior to incorporation into riboflavin.

A source of the 4-carbon unit, unlike an active acetyl compound, has been found in studies with enzyme preparations from *E. coli* and *Ashbya gossypii* (62). In these incubation mixtures, only 6,7-dimethyl-8-ribityllumazine, a

⁴ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH₂), for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH₂), for triphosphopyridine nucleotide, reduced form (TPNH).

suitable buffer, and enzyme were required to obtain net formation of riboflavin. Purification of the activity up to 100-fold from extracts of *A. gossypii* disclosed no requirement for an additional medium component. Stimulation of activity was not observed upon the addition, either alone or in various combinations, of the following materials: acetoin, 2,3-butanedione, DL- β -hydroxybutyrate, pyruvate, acetate, acetyl phosphate, acetyl CoA, ribose-5-phosphate, glucose-6-phosphate, ATP, ADP, NAD, NADH₂, NADP, NADPH₂, CoA, or Mg⁺⁺. Radioactivity from acetate-C¹⁴ or glucose-C¹⁴ was not incorporated into riboflavin with either crude or purified preparations of the organisms, although net synthesis of the vitamin did occur. These results implied that 6,7-dimethyl-8-ribityllumazine supplies not only the ribitylpteridine portion of riboflavin but also the remaining four carbon atoms to complete the *o*-xylene ring. Additional studies clarified the reaction. If ribo-

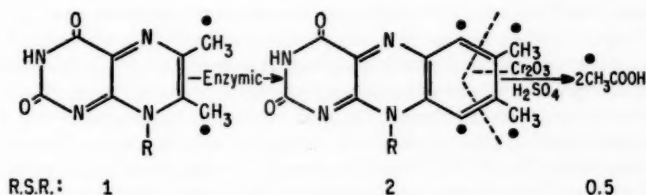


FIG. 5. Position of label in riboflavin formed from 6,7-dimethyl-C¹⁴-8-ribityllumazine.

R.S.R. = relative molar specific radioactivity; • = C¹⁴

flavin were formed by condensation of 6,7-dimethyl-8-ribityllumazine with four carbon atoms from an extraneous source, one molecule of pteridine would be consumed for each mole of flavin formed in the reaction (see Fig. 3). Alternatively, if the ribitylpteridine acted as both acceptor and donor of the four carbons, the ratio of substrate disappearance to product formation should be between 2 and 3. The experimentally observed ratio was 2.3, thus supporting the latter mechanism. It would appear reasonable that the four carbon atoms in such a mechanism would originate from the methyl groups and carbons 6 and 7 of 6,7-dimethyl-8-ribityllumazine (Fig. 5). Hence, the conversion of 6,7-dimethyl-C¹⁴-8-ribityllumazine to riboflavin and the chemical degradation of the latter by chromic acid oxidation to acetic acid (methyl groups plus carbons 6 + 7) should result in a ratio of molar specific radioactivities of 6,7-dimethyl-C¹⁴-8-ribityllumazine:riboflavin:acetate of 1:2:0.5 (Fig. 5); an experimental value for this ratio of 1:2.2:0.33 was obtained in an experiment with purified enzyme from *Ashbya gossypii* (62). Further proof of the mechanism would come with the identification of a product resulting from the removal of a two-carbon or four-carbon unit from 6,7-dimethyl-8-ribityllumazine. Such a substance has not yet been identified.

Sund & Little (81) made the interesting observation that production of

riboflavin by *E. ashbyii* was markedly depressed by concentrations of 3-amino-1,2,4-triazole which did not inhibit growth. Similarly, corn and pea leaf tissues treated with this compound were albinistic and had a greatly lowered riboflavin content (81). It would be interesting to know the step at which aminotriazole inhibits riboflavin biogenesis. It does not affect the conversion of 6,7-dimethyl-8-ribityllumazine to the vitamin by a purified enzyme from *A. gossypii* (55).

The accumulated evidence indicates that riboflavin synthesis starts from the pyrimidine, rather than the benzenoid, end of the molecule. Thus, it has been shown with growing cultures of *A. gossypii* that (a) label from glycine- N^{15} is recovered in the pyrazine and not the pyrimidine ring of the vitamin (10); (b) N^{15} from uniformly labeled purine is transferred as a unit to riboflavin by cells of *E. ashbyii* (21); and (c) 6,7-dimethyl-8-ribityllumazine is probably an intermediate. A precursor-product relationship could not be demonstrated by the use of compounds related to the aromatic ring portion of riboflavin in the medium of ascomycetes (4, 12, 16, 26), although flavin production has been occasionally inhibited. However, 1,2-dimethyl-4-amino-5-(α -1'-ribitylamino) benzene has been reported to be converted to riboflavin by *Mycobacterium tuberculosis* (63), and both this compound and 4,5-dimethylphenylenediamine are claimed to have a trace of riboflavin activity for various bacteria (64, 65). Thus, 1,2-dimethyl-4,5-diaminobenzene and 1,2-dimethyl-4-amino-5-ribitylamino benzene were, respectively, 1/100,000 and 1/10,000 as active as riboflavin for *L. casei* (65). In an organism requiring vitamin B_2 (66) but not vitamin B_{12} for growth, the growth effects reported cannot be attributed conclusively to a precursor function of these substances. The ineffectiveness of these materials for riboflavin synthesis is in marked contrast to the ease with which a great number of phenyldiamines and imidazole derivatives are utilized in the formation of a series of vitamin- B_{12} compounds by many species of bacteria (see p. 435). These results imply that the benzimidazole moiety of dimethylbenzimidazole cobalamide and ring A of riboflavin arise by completely different metabolic routes, but the variance in the *de novo* formation of the two vitamins might occur prior to 6,7-dimethyl-8-ribityllumazine. There is as yet no evidence that 1,2-dimethyl-4,5-diaminobenzene or dimethylbenzimidazole per se are generated as intermediates during the biogenesis of vitamin B_{12} .

6-Methyl-7-hydroxy-8-ribityllumazine.—This compound, fluorescing blue (violet), has been isolated from *Eremothecium ashbyii* (39, 67). *Ashbya gossypii* (56, 68) and *Clostridium acetobutylicum* (44). A substance with this configuration was synthesized by Plaut & Maley (56, 68) (Fig. 2) and shown to be identical with material from *A. gossypii* and Masuda's compound V. After some uncertainty (39, 67), there is now general agreement on its structure [Masuda *et al.* (69, 70); McNutt (71)].

A related metabolic origin for this pteridine and 6,7-dimethyl-8-ribityllumazine and riboflavin has been shown with cultures of *E. ashbyii* and *A. gossypii*, in which extensive transfer of radioactivity from uniformly C^{14}

labeled adenine and guanine into the pyrimidine moiety and of formate-C¹⁴ into carbon 2 were observed (68, 71, 72).

Taylor & Loux (73) have synthesized two compounds related in structure: 2-amino-4-hydroxy-8-(D-1'-ribityl)-7,8-dihydropteridine-6-carboxylic acid and 2-amino-4-hydroxy-7-keto-8-(D-1'-ribityl)-7,8-dihydropteridine-6-carboxylic acid.

The origin of carbons 6+7 of pteridines (carbons 8a+10a of riboflavin).—Information gathered concerning the biosynthesis of riboflavin and the 8-ribityl-2,4-pteridiones indicates that a derivative of 4,5-diamino-2,6-dihydroxypyrimidine condenses with another fragment to form a pteridine. Brown *et al.* (15) tested the effect of 4,5-diamino-2,6-dihydroxypyrimidine on flavin production with *E. ashbyii*; no enhancement was found, but the added compound may not be able to penetrate to the site of conversion. However, indirect evidence supports the possibility that 4,5-diamino-2,6-dihydroxypyrimidine is an intermediate. Goodwin & Treble (74) reported that the addition of glyoxal to growing cultures of *E. ashbyii* led to the formation of the expected chemical condensation product, lumazine. Furthermore, radioactivity from added 3-hydroxy-2-butanone-1-C¹⁴ was incorporated into ring A of riboflavin exclusively (75). One-half of the radioactivity of the *o*-xylene moiety was in the methyl groups, none in carbons 6 and 7, and the remainder, in analogy with previous experiments with acetate-C¹⁴ and glucose-C¹⁴ (61), presumably in carbons 5 and 8. The labeled acetoin was incorporated more efficiently into ring A than acetate-2-C¹⁴ (75).

Masuda proposed that biogenesis of 8-ribityl-2,4-pteridiones occurs by the condensation of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine with 2,3-butanedione (or acetoin) (38) or pyruvate (76) to form, respectively, 6,7-dimethyl-8-ribityllumazine or 6-methyl-7-hydroxy-8-ribityllumazine as in the chemical synthesis of these substances (Fig. 2). Masuda's theory is supported by Kishi *et al.* (77) and Katagiri *et al.* (46, 51, 57), who showed that the formation of 6,7-dimethyl-8-ribityllumazine from 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and acetoin required the presence of an extract from certain microorganisms (*Eremothecium ashbyii* and *Aerobacter aerogenes*). The enzyme preparation appears to effect the well-known (78) oxidation of acetoin to 2,3-butanedione. The latter condenses readily with the pyrimidine-orthodiamine, and the reaction appears to be non-enzymic. This may explain the lack of specificity of the reaction whereby 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine can be effectively replaced by 4,5-diamino-2,6-dihydroxypyrimidine, 4-methylamino-5-amino-2,6-dihydroxypyrimidine, and 4-(2'-hydroxyethyl)amino-5-amino-2,6-dihydroxypyrimidine, leading to the formation of 6,7-dimethylumazine, 6,7,8-trimethylumazine, and 6,7-dimethyl-8-(2'-hydroxyethyl)umazine, respectively. The mechanism has much to recommend it; it has the advantage of simplicity, and the formation of the required substrate, acetoin, has been demonstrated (38). However, the other substrate required, 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine, has not been demonstrated in the organism. In fact, Goodwin & Treble (74) re-

covered lumazine and not 8-ribityllumazine, the product expected from the above mechanism, upon the addition of glyoxal to *E. ashbyii*.

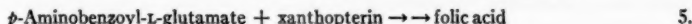
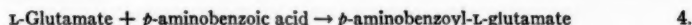
Masuda's proposal that 6-methyl-7-hydroxy-8-ribityllumazine arises from the condensation of 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine and pyruvate—as in the chemical synthesis (Fig. 2)—has been supported by rather indirect biological evidence. Thus, the addition of large quantities of pyruvate to growing cultures of *E. ashbyii* by Masuda *et al.* (76) enhanced the formation of 6-methyl-7-hydroxy-8-ribityllumazine and decreased riboflavin production. This was interpreted as a competition of pyruvate and acetoin for available 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine (see Fig. 2), increased amounts of pyruvate leading to the formation of more 6-methyl-7-hydroxy-8-ribityllumazine and less 6,7-dimethyl-8-ribityllumazine, the latter resulting in lower yield of riboflavin. However, preliminary experiments in this laboratory (79) indicate that the addition of pyruvate-1- C^{14} to growing cultures of *A. gossypii* produces no radioactivity in the methyl group and the carbon 6 and 7 moiety of 6-methyl-7-hydroxy-8-ribityllumazine isolated from the fermentation; some radioactivity did appear in this portion of the molecule when pyruvate-3- C^{14} was used. The evidence gathered in enzyme experiments that 6-methyl-7-hydroxy-8-ribityllumazine may arise from 6,7-dimethyl-8-ribityllumazine (49, 80) needs clarification, since the conversion also occurs chemically under the conditions used. The non-enzymic formation of 6-methyl-7-hydroxy-8-ribityllumazine and other products from 6,7-dimethyl-8-ribityllumazine has been noted, particularly with solutions of the radioactive compound (41). The loss of the methyl group in position 7 and its replacement by a hydroxyl is probably an oxidative or a peroxidative process, since decomposition of 6,7-dimethyl-8-ribityllumazine is facilitated by aeration or by addition of H_2O_2 (23, 52).

The nature of the direct precursors of carbons 6 and 7 and of the substituents on these carbons in other pteridines is even more uncertain than in the case of the 8-ribitylpteridiones. At present even the extent to which the metabolism of this portion of the molecule is related in various pteridines is uncertain. Some genetic information has been accumulated in *Drosophila*, and it supports such connections for compounds like biopterin, sepiapterin, compound A, and drosopterins (114).

Weygand *et al.* (82) have reported that the administration of glucose-1- C^{14} to the butterfly *Pieris brassicae* yielded leucopterin containing 60 per cent of the radioactivity of the molecule in carbons 6 and 7; the remainder probably was in carbon 4.

The related xanthopterin has been reported by Korte *et al.* (83, 84) and Katunuma *et al.* (85, 86) to serve as a precursor of certain pteric acid derivatives. Xanthopterin can partially replace folic acid in the nutrition of certain forms of life, but this sparing action has been attributed to an inhibition of destruction of the vitamin (87). Katunuma *et al.* (85, 86) demonstrated a net formation of folic acid from *p*-aminobenzoic acid, L-glutamate, and xanthopterin; it appeared that *p*-aminobenzoic acid and L-glutamate

reacted to form *p*-aminobenzoyl-L-glutamate prior to condensation with xanthopterin:



Korte *et al.* (83, 84) studied the incorporation of radioactivity from xanthopterin-8a-C¹⁴ or *p*-aminobenzoate-2,6-C¹⁴ into various pteroyl compounds with growing cells or extracts from *Enterococcus stei*, *E. coli*, *Lactobacillus plantarum* 10S, *Streptococcus faecalis* R, and *Pichia membranaefaciens* (Fig. 6). Incorporation occurred in all cases; interesting differences were noted in the labeled compounds formed, depending on the substance added and the nature of the preparation of microorganisms. The results are summarized in Table I. Addition of xanthopterin-8a-C¹⁴ to growing cultures (83) gave rise to N⁵-formyltetrahydropteroate, whereas *p*-aminobenzoate-2,6-C¹⁴

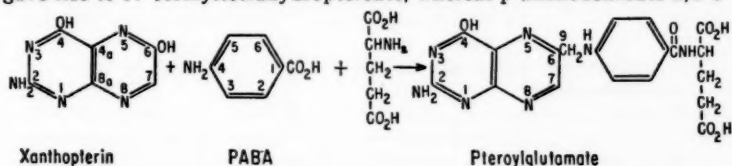


FIG. 6. Formation of pterates from xanthopterin and *p*-aminobenzoate (PABA).

yielded a number of labeled glutamate-containing pterates. Of these organisms, *L. plantarum* 10S is the only one purportedly capable of utilizing *p*-aminobenzoyl-L-glutamate for folic acid synthesis (88, 89), and the addition of this substance with xanthopterin-8a-C¹⁴ led to labeled pterates containing glutamate (Table I). Korte & Synnatschke (84) concluded, therefore, that added xanthopterin inhibits the enzyme that catalyzes the condensation of *p*-aminobenzoate or of pterate with glutamate. However, this mechanism seems inconsistent with their experiments, since radioactivity from xanthopterin-8a-C¹⁴ added to extracts of these same organisms was recovered in pterates containing glutamate. It may be that xanthopterin does not inhibit the utilization of glutamate in the condensation reaction but interferes with the penetration of glutamate into intact cells. Korte & Synnatschke (84) believe that xanthopterin is not an intermediate in biogenesis of pteroylglutamates since radioactivity from this compound was utilized poorly in the formation of such derivatives by intact cells.³

Whether or not xanthopterin is a true intermediate, the results have interesting implications. The transfer of label from xanthopterin-8a-C¹⁴ to pterates in extracts of *S. faecalis* R (84) is a notable observation, since this bacterium is known to require pteric acid derivatives for growth (90); the rate of synthesis in the organism appears to be suboptimal for growth but detectable by the more sensitive radioisotope method. The results of Korte *et al.*

TABLE I*

INCORPORATION OF C¹⁴ FROM XANTHOPTERIN-8a-C¹⁴ AND *p*-AMINO BENZOATE-2,6-C¹⁴ INTO PTEROATES BY VARIOUS PREPARATIONS

Radioactive compound added	Microorganisms	Nature of preparation	Radioactive compounds recovered†
Xanthopterin-8a-C ¹⁴	<i>E. stei</i> , <i>E. coli</i> , <i>L. plantarum</i> 10S, <i>P. membranaefaciens</i> , <i>S. faecalis</i> R	Growing cells	N ⁶ -fPH ₄
<i>p</i> -aminobenzoic acid-2,6-C ¹⁴	<i>E. stei</i> , <i>E. coli</i> , <i>S. faecalis</i> R, <i>P. membranaefaciens</i>	Growing cells	N ⁶ -fPGH ₄ , N ⁶ -fPDGH ₄ , N ⁶ -fPTGH ₄
Xanthopterin-8a-C ¹⁴	<i>E. stei</i> , <i>S. faecalis</i> R, <i>E. coli</i> , <i>P. membranaefaciens</i>	Extracts	PG, N ⁶ -fPGH ₄ , N ⁶ -fPDGH ₄
Xanthopterin-8a-C ¹⁴ + <i>p</i> -aminobenzoyl-L-glutamate	<i>L. plantarum</i> 10S	Growing cells	N ⁶ -fPH ₄ , N ⁶ -fPDGH ₄

* Data from Korte, F., and Synnatschke, G., *Ann. Chem. Liebigs*, **628**, 153 (1959); Korte, F., Barkemeyer, H., and Synnatschke, G., *Z. physiol. Chem.*, **314**, 106 (1959).

† N⁶-fPH₄, N⁶-formyl tetrahydropteratoate; N⁶-fPGH₄, N⁶-formyl tetrahydropteroyl glutamate; N⁶-fPDGH₄, N⁶-formyl tetrahydropteroyldiglutamate; N⁶-fPTGH₄, N⁶-formyl tetrahydrotriglutamate; PG, pteroylglutamate.

and Katunuma *et al.* raise the question of the origin of carbon 9, which is not present in the xanthopterin precursor (Fig. 6). This carbon may arise from a one-carbon derivative, e.g., carbon dioxide, formate, formaldehyde, or methyl. Katunuma *et al.* (86) indicated that the formation of folic acid from *p*-aminobenzoylglutamate and xanthopterin required cocarboxylase or biotin, or both, as cofactors, but the latter were not necessary when 2-amino-4-hydroxypteridine-6-carboxylic acid was used instead of xanthopterin. These results seem to imply carboxylation of 2-amino-4,6-dihydroxypteridine by carbon dioxide to yield 2-amino-4-hydroxypteridine-6-carboxylic acid. Incorporation of radioactivity from formate-C¹⁴ into 6-methyl-7-hydroxy-8-ribityllumazine indicates that the major labeling occurs in carbon 2 (68); no trace of radioactivity could be found in the methyl group (carbon 9) even upon direct degradation (79). The basic configuration of this compound is very similar to that of the pteridine moiety of pterates (compare Fig. 2 and Fig. 6); however, there is no evidence that the analogous 3-carbon moieties of these pteridines share a common metabolic origin.

Enzymic formation of pteroyl derivatives.—The formation of folic acid-like components from *p*-aminobenzoate, glutamate, and an endogenous precursor by resting cells was first noted by Nimmo-Smith, Lascelles & Woods (91, 92);

TABLE II

EFFECT OF VARIOUS PTERIDINES ON THE ENZYMIC FORMATION OF MATERIALS WITH FOLIC ACID ACTIVITY FOR *STREPTOCOCCUS FAECALIS* R

Pteridine	Activity	Organism	Reference
2-Amino-4-hydroxy-6-trihydroxypropyl-pteridine (oxidized and reduced)	none	<i>Lactobacillus arabinosus</i>	(93)
2-Amino-4-hydroxy-6-carboxypteridine	none	<i>L. arabinosus</i> and <i>E. coli</i>	(93, 94)
2-Amino-4-hydroxy-6-carboxypteridine	active	<i>Mycobacterium avium</i>	(86)
2-Amino-4,6-dihydroxypteridine (xanthopterin)	none	<i>L. arabinosus</i> and <i>E. coli</i>	(93, 94)
2-Amino-4,6-dihydroxypteridine (xanthopterin)	active	<i>M. avium</i> , <i>E. stei</i> , <i>E. coli</i> , <i>S. faecalis</i> R, <i>L. plantarum</i> 10S, <i>P. membranaefaciens</i>	(84, 85)
2-Amino-4-hydroxy-6-pteridine carbox-aldehyde	active	<i>L. arabinosus</i> and <i>E. coli</i>	(93, 94)
2-Amino-4-hydroxy-6-pteridine carbox-aldehyde (reduced)	very active	<i>L. arabinosus</i>	(93)
2-Amino-4-hydroxy-6-pteridine carbox-aldehyde (reduced)	none	<i>E. coli</i>	(95)
2-Amino-4-hydroxy-6-hydroxymethyl-pteridine	none	<i>L. arabinosus</i> and <i>E. coli</i>	(93, 94)
2-Amino-4-hydroxy-6-hydroxymethyl-pteridine (reduced)	very active	<i>L. arabinosus</i> and <i>E. coli</i>	(93, 95)

the addition of 2-amino-4-hydroxy-6-pteridine aldehyde increased the yield of the folic acid-like material. A number of investigators have studied the synthesis of pteric acid compounds with extracts of microorganisms. The findings of Korte *et al.* (83, 84) and Katunuma *et al.* (85, 86) using xanthopterin as the precursor of the pteridine moiety have already been discussed.

Shiota (93) developed folic acid activity for *S. faecalis* R by using extracts from *L. arabinosus* in a system that contained *p*-aminobenzoate or *p*-aminobenzoyl-L-glutamate. The enzymic activity was lost upon dialysis but was restored by the addition of heated cell extract, which may contribute a precursor pteridine since it could be replaced by a number of synthetic pteridines (Table II). Among these, 2-amino-4-hydroxy-6-pteridine aldehyde showed activity which was increased upon reduction of the compound with zinc and acid. Brown (94) also found this pteridine active with an extract from *E. coli* but reported (95) the reduced form to be inactive. Neither investigator detected folic acid activity with added xanthopterin [cf. Korte & Synnatschke (84); Katunuma *et al.* (85, 86)], 2-amino-4-hydroxy-6-car-

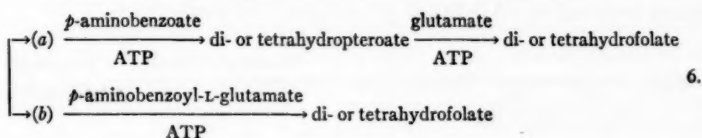
boxypteridine [cf. Katunuma & Shoda (86)], or the oxidized form of 2-amino-4-hydroxy-6-hydroxymethylpteridine; but the reduced form of the 6-hydroxymethyl compound was very effective (93, 95). Shiota (93) has studied the latter in detail; it is probably a tetrahydropteridine since the catalytic reduction of 2-amino-4-hydroxy-6-hydroxymethylpteridine was accompanied by an uptake of four atoms of hydrogen per mole of compound. It remains to be proven whether this compound per se is the actual reactant in the enzyme system and whether it is identical with the endogenous substrate. Attempts to show reduction of 2-amino-4-hydroxy-6-hydroxymethylpteridine by the inclusion of NADH_2 and NADPH_2 in the complete system were unsuccessful (93). Brown (94) has reported enhanced synthesis of "folic acid" in the presence of NADH_2 and has claimed (95) that the effect of this coenzyme can be partially replaced by reduced 2-amino-4-hydroxy-6-hydroxymethylpteridine, although it is not stated what pteridine was used with NADH_2 in this comparison. ATP seems to be needed in all systems, but the mechanism of its participation is not clear. Activation of the pteric acid derivative or of *p*-aminobenzoic acid might be necessary before condensation with L-glutamate, analogous with the incorporation of amino acids into peptide structures, and Katunuma *et al.* (85, 86) claim that *p*-aminobenzoyladenylate and *p*-aminobenzoyl CoA are intermediates in the synthesis of *p*-aminobenzoyl-L-glutamate. The formation of the latter appears necessary for condensation with a pteridine to make "folic acid" in *Mycobacterium avium*. The sequence of reactions in *L. arabinosus* and *E. coli* is less certain. Materials chromatographically similar to tetrahydrofolate, dihydrofolate, and folate were formed by extracts of *L. arabinosus* in the presence of 2-amino-4-hydroxy-6-hydroxymethyl tetrahydropteridine, *p*-amino-benzoyl-L-glutamate, and ATP; however, a substance resembling pteric acid was formed when *p*-aminobenzoyl-L-glutamate was replaced by a mixture of *p*-aminobenzoate and L-glutamate. Brown has stated in his review (95):

A comparison of the effectiveness with which *p*-aminobenzoate and *p*-aminobenzoyl-glutamate were used as substrate to yield pteric acid and folic acid, respectively, showed that in the *E. coli* system *p*-aminobenzoate was used about 40 times more effectively than *p*-aminobenzoylglutamate.

However, it is not stated whether or not a pteroylglutamate is formed from the pterate in the system containing glutamate and *p*-aminobenzoate.

These results leave considerable uncertainty about the nature of the substrates, intermediates, and products in this series of reactions. Some of the variations may be attributable to the different microorganisms used and others to differences in experimental details. This field is still in the early stages of development, but the results obtained by Shiota (93) and Brown (94, 95), e.g., with 2-amino-4-hydroxy-6-hydroxymethyltetrahydropteridine, the beneficial effects of reducing conditions on the over-all reaction, and the detection of certain di- and tetrahydropteroyl compounds as products, may indicate the following biosynthetic pathway:

2-Amino-4-hydroxy-6-hydroxymethyl tetrahydro(dihydro?)pteridine \rightarrow



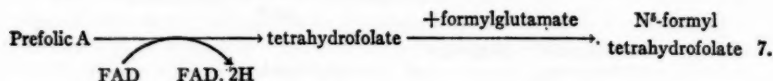
Jaenicke & Chan (237) have reported recently that a soluble enzyme from *E. coli* or yeast catalyzes the conversion of 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine to dihydrofolate.

The availability of enzyme preparations capable of synthesizing derivatives of pteroylglutamate has renewed interest in the mechanism of the well-known (97) antagonism between *p*-aminobenzoate and sulfanilamide derivatives. Katunuma (96) [as quoted by Brown (95)] found that sulfanilamide inhibits the condensation of *p*-aminobenzoate with L-glutamate in *M. avium*; Brown (95) believes the site of inhibition in *E. coli* is in the formation of a pterate from pteridine and *p*-aminobenzoate. Katunuma & Shoda (86) have localized the inhibition of folic acid synthesis in extracts of *M. avium* by *p*-aminosalicylic acid at the steps involved in the condensation of xanthopterin and *p*-aminobenzoylglutamate, at a stage of the reaction sequence similar to where Brown (95) reported that sulfa drugs act in *E. coli*. Not enough data are available to conclude whether the sulfa drugs inhibit several steps in the synthesis of pteroylglutamates or whether variations occur because of differences in the mode of formation of folates in various species. *p*-Aminobenzoate has usually been more effective than *p*-aminobenzoyl-L-glutamate in reversing sulfanilamide inhibition of growth in most microorganisms; only in Auhagen's results with *Lactobacillus plantarum* 10S did the reverse appear to be the case [see (98)]. Korte & Synnatschke (84) also encountered species differences in the incorporation of xanthopterin-8a-C¹⁴ into various pteroyl compounds in the presence of *p*-aminobenzoylglutamate or *p*-aminobenzoate (Table I), and it is notable that both *M. avium* and *L. plantarum* 10S differed from other organisms tested in the nature of the radioactivity-containing pterates that were recovered.

Interconversion of derivatives of pteroylglutamate.—The interrelationships of these compounds and their involvement in one-carbon transfer reactions have been reviewed (99 to 101) and will not be discussed in detail here.

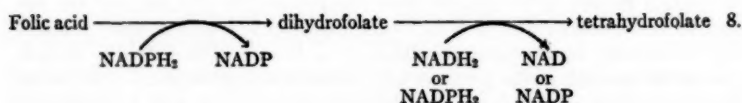
Donaldson & Keresztesy (102) have examined extracts from unautolyzed liver for unknown folic acid-active materials. One partially purified material has been called prefolic A. Prefolic A does not support the growth of *Streptococcus faecalis* R and *Leuconostoc citrovorum* 8081, but activity developed upon incubation of the material with a swine liver enzyme preparation. The product of the purified conversion enzyme was tetrahydrofolic acid which could be transformed to N⁶-formyltetrahydropteroylglutamate upon the addition of formylglutamate and N⁶-formyltetrahydropteroylglutamate-glu-

tamate-transformylase. The initial conversion of prefolic A to tetrahydrofolate involved an FAD-linked dehydrogenation step.



FAD could not be replaced by riboflavin, flavin mononucleotide, or pyridine nucleotides in this reaction. Inhibitors of the reduction of folic acid to tetrahydrofolic acid, such as aminopterin and amethopterin (103), as well as 2,6-dichloro-amethopterin and Daraprim, did not inhibit the reaction. The data suggest that prefolic A is a reduced form of tetrahydrofolate, but the site of oxidation of the prefolic A molecule to tetrahydrofolate is not known. The physiological significance of prefolic A remains to be evaluated. However, it is of interest that the growth-promoting activity for *S. faecalis* R and *L. citrovorum* 8081 of a hot water extract of horse liver was only 2 per cent of that of the extract of autolyzed tissue (104).

The involvement of a flavoprotein in the conversion of prefolic A to tetrahydrofolate is of interest in connection with the claim (105) that a sheep liver enzyme requires flavin mononucleotide as well as NADPH_2 for the conversion of folate to tetrahydrofolate. Dialysis of this preparation against $\text{H}_2\text{O}-\text{CO}_2$ reduced the activity to 67 per cent, and addition of $10^{-4}M$ flavin mononucleotide restored the activity to 84 per cent of that prior to dialysis. Atebrine is reported to depress the reduction of folate. Peters & Greenberg [(106); see also Futterman (107)] previously obtained pyridine nucleotide-linked folic acid reductase from this source; the reaction proceeded in two steps:



A similar folic acid reductase has been purified eighteenfold from chicken liver extract by Zakrzewski (108); in this case, it appears that a single enzyme catalyzes the reduction of folate and dihydrofolate, and a number of substances were tested as substrates. N^{10} -Methyl-folate, folate, diglutamyl folate, pteridine-6-aldehyde, pteroate, 9-methyl folate, and perhaps 9,10-dimethylfolate were reduced by NADPH_2 , whereas N^{10} -formyl-folate, pteridine-6-carboxylate, 6-methylpteridine, and xanthopterin were not. No relationship seemed to exist between the biological activity of the compounds and their ability to be reduced by the enzyme preparation.

An enzyme has been purified from chicken liver (109) which carries out the isomerization of N^5 -formyltetrahydrofolate to N^{10} -formyltetrahydrofolate



No evidence could be obtained that N^6, N^{10} -methenyltetrahydrofolate is an intermediate in this reaction.

Wood & Hitchings (110) have shown that 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (Pyrimethamine, Daraprim) is a non-competitive inhibitor for the conversion of folate to N^5 -formyltetrahydrofolate in extracts of *S. faecalis* R; the concentration of drug required for inhibition in the extract was of the same order of magnitude as that required for inhibition of growth of the organism. However, a comparison of the conversion activity of extracts from Pyrimethamine-resistant and non-resistant strains of *S. faecalis* showed that equivalent sensitivity to the drug was retained. Evidence with whole cells suggested that the pathway to a conjugated form of N^5 -formyltetrahydrofolate may be involved in the resistance to this drug (111). This substance did not inhibit folic acid synthesis in *E. coli* (110).

THIAMINE

BIOGENESIS

The formation of thiamine from pyrimidine and thiazole.—The pyrimidine and thiazole portions of thiamine can replace the intact vitamin molecule to support growth of a number of microorganisms [see (115) for review]. These organisms can be divided into four groups according to the moiety of the thiamine molecule (aside from the vitamin itself) that will satisfy their growth requirement: (a) thiamine only, (b) thiazole (4-methyl-5-(β -hydroxyethyl)thiazole), (c) pyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine), and (d) pyrimidine plus thiazole.

A number of thiamine-requiring strains of *Neurospora crassa* differ in quantitative growth response to the vitamin and its pyrimidine and thiazole components (115, 116). Eberhart & Tatum (117) have crossed one of these mutants, the growth of which could also be supported by a combination of the thiazole and the pyrimidine, with a wild type. A number of thiamine-requiring strains were isolated from the cross; these strains had about the same quantitative responses to thiamine as the thiamineless parent but without the ability to grow on thiazole and pyrimidine, either alone or in combination. The offspring, like the parent strain, could absorb about the same amount of thiamine from the medium into cells (measured as "bound" thiamine); but the crosses, when grown on a medium with limiting vitamin B₁, were unable to produce significant amounts of thiamine when exposed to a combination of the thiazole and the pyrimidine. The "wild strain" appears to contribute a gene (*thi-10*) which depresses synthesis of thiamine from the thiazole and the pyrimidine. While this effect could only be observed with the crosses of one mutant strain (gene *thi-1*) having a high thiamine requirement, a modification of the ability to synthesize thiamine from thiazole and pyrimidine was also observed with the offspring when other thiamineless mutants were crossed with the gene *thi-10*-containing strain, although their growth requirements were unchanged. Since a part of the mechanism of thiamine biosynthesis has now been elucidated (see below), it will be inter-

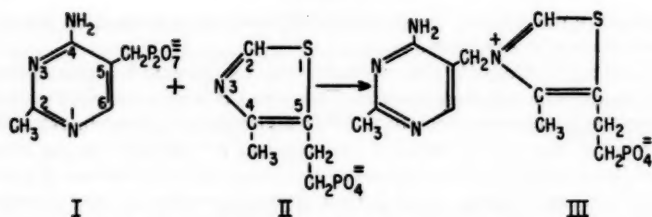
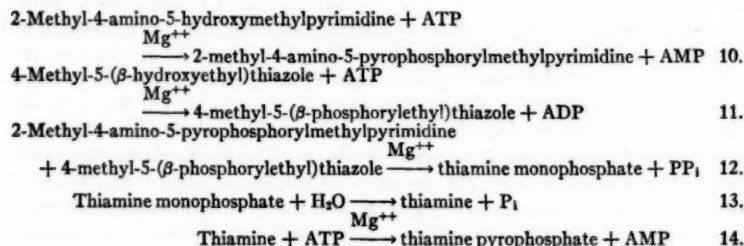


FIG. 7. Formation of thiamine monophosphate.

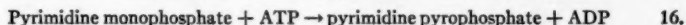
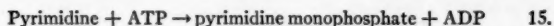
esting to know whether any specific enzyme in the sequence of reactions is affected by gene *thi-10*.

Harris & Yavit (118) reported that the formation of thiamine from the pyrimidine and the thiazole by bakers' yeast extracts required ATP and Mg^{++} . Substitution of the pyrimidine monophosphate for the pyrimidine appeared to abolish the ATP requirement. However, with more purified enzyme, Leder (119) observed that ATP was still required even in the presence of 2-methyl-4-amino-5-phosphorylmethylpyrimidine and that the product of the condensation reaction was thiamine monophosphate rather than free thiamine. A number of workers have reached essentially the same conclusions about the mechanism [Leder (120); Camiener & Brown (121 to 123); Nose *et al.* (124); Suzuoki & Kobata (125)]; namely, the pyrimidyl pyrophosphate (I) and the thiazole phosphate (II) condense to form thiamine monophosphate (III) (Fig. 7). The formation of cocarboxylase from the pyrimidine and the thiazole is thought to occur by way of the following series of reactions:



In the formation of thiamine monophosphate (Reaction 12) by purified enzyme preparations, pyrimidyl pyrophosphate and thiazole monophosphate were superior as substrates to pyrimidyl monophosphate and thiazole pyrophosphate or to the non-phosphorylated compounds. Nose *et al.* (124) and Leder (120) synthesized the phosphorylated substrates by the method of Weijlard (126); Camiener & Brown (121, 122) isolated these substances from enzymic reaction mixtures and tested them with various enzyme preparations.

Complete separation of the individual enzymes that catalyze the reactions represented in Equations 10 to 13 has not been reported so far; hence, there is still uncertainty about the precise stoichiometry of some steps. Camiener & Brown (123) obtained two fractions from yeast extract: Fraction 1 phosphorylated the pyrimidine and thiamine, and Fraction 2 phosphorylated the thiazole and contained thiamine monophosphate synthetase. Neither fraction had the activity (present in the crude extract) that degrades thiamine monophosphate to thiamine. Incubation of the pyrimidine and ATP with Fraction I led to the formation of the pyrimidine pyrophosphate and the pyrimidine monophosphate (122). The pyrimidine pyrophosphate may first be formed as represented in Equation 10, followed by the action of a contaminating pyrophosphatase yielding the pyrimidine monophosphate. Alternately, the pyrimidine pyrophosphate might be formed by two kinase reactions in which the pyrimidine monophosphate would be an intermediate:



Attempts to distinguish between these possibilities (122) were unsuccessful because of contamination of the preparation with phosphatases and adenylate kinase. The stoichiometry of ATP disappearance and ADP formation has not been reported, but the thiazole phosphate is probably formed as represented in Equation 11 since thiazole pyrophosphate has not been detected in the reaction mixtures. The pyrimidine monophosphate will not replace pyrimidine pyrophosphate in the thiamine monophosphate synthetase reaction (Equation 12) (120, 123, 124); hence, inorganic pyrophosphate, as well as thiamine phosphate, should be a product of this condensation. The formation of pyrophosphate was not demonstrated, and an attempt to pyrophosphorolyze thiamine monophosphate has not been reported; however, added inorganic pyrophosphate did inhibit the formation of thiamine monophosphate (121). The properties of thiamine monophosphate phosphatase (Equation 13) have not been studied in detail. It appears certain that the substrate preceding cocarboxylase is free thiamine (Eq. 9) rather than thiamine monophosphate (123).

Pyrimidine.—It has been reported that a thiamineless strain of *E. coli* which is capable of growth in the presence of the pyrimidine and the thiazole, could also utilize a combination of thymine and uracil instead of the pyrimidine (127). The biogenesis of 2-methyl-4-amino-5-hydroxymethylpyrimidine from simple precursors has not been further investigated. However, structural analogues of this pyrimidine have been tested for their ability to participate in thiamine biosynthesis. Nakayama (128) has studied the ability of analogues of 2-methyl-4-amino-5-hydroxymethylpyrimidine with the hydroxymethyl group at position 5 replaced by other substituents to support growth. 2-Methyl-4-amino-5-aminomethylpyrimidine and 2-methyl-4-amino-5-formylpyrimidine were as effective as the 5-hydroxymethyl compound; however, 2-methyl-4-amino-5-cyanopyrimidine and 2-methyl-4-amino-5-

carboxypyrimidine inhibited growth. Schopfer (129) had shown previously that growth of *Rhodotorula rubra* was enhanced by substituting 2-methyl-4-amino-5-aminomethylpyrimidine or 2-methyl-4-amino-5-(N-thioformyl-aminomethyl)pyrimidine for the required vitamin B₁; the 5-cyano derivative was inactive. To determine whether the growth promotion of 2-methyl-4-amino-5-aminomethylpyrimidine is correlated with its action as a precursor of the pyrimidine moiety of thiamine, Korte & Weitkamp (130) studied the incorporation of radioactivity from 2-methyl-4-amino-5-aminomethyl-C¹⁴ pyrimidine into thiamine with a number of microorganisms. Radioactivity from this added pyrimidine was recovered in thiamine from the various organisms regardless of whether they were thiamine autotrophs or could grow with the pyrimidine alone, the thiazole alone, or required a combination of the two. However, the thiazole-requiring strains used (*Phycomyces blakesleeanus*, *Mucor ramannianus*) could not have had an absolute need for this component, since enough growth must have been obtained to isolate radioactive thiamine from the organisms raised in a medium (129) containing glucose, asparagine, salts, and radioactive 2-methyl-4-amino-5-aminomethylpyrimidine but no added thiazole. It is doubtful whether intact 2-methyl-4-amino-5-aminomethylpyrimidine is utilized for thiamine synthesis, since Camiener & Brown (122) found that this compound and 2-methyl-4-amino-5-methoxymethylpyrimidine were enzymically converted to 2-methyl-4-amino-5-hydroxymethylpyrimidine by yeast extracts prior to utilization in the synthesis of vitamin B₁. 2-Methyl-4-amino-5-bromopyrimidine was also active in their system, and it was presumed that the bromo group was similarly replaced by a hydroxyl group.

Thiazole.—Nakayama (127) examined the growth of thiamineless *E. coli* mutants with compounds substituted on the thiazole ring. The hydrogen atom at position 2 was replaceable by a sulfur, and the double bond in positions 2 and 3 could be reduced without loss of growth-promoting activity. However, substitution of a hydroxyl or an amino group at carbon 2 destroyed the activity. Indeed, 2-amino-4-methyl-5-(β -hydroxyethyl)thiazole was reported to inhibit completely the growth of the "wild" strain of *E. coli* ATCC 9637 at a medium concentration of $10^{-7}M$ (131). This inhibition was antagonized competitively by 4-methyl-5-(β -hydroxyethyl)thiazole ($10^{-9}M$) or by 2-methyl-4-amino-5-hydroxymethylpyrimidine ($10^{-6}M$); thiamine produced a non-competitive reversal. Thus, it appears that the 2-amino-4-methyl-5-(β -hydroxyethyl)thiazole blocks some stage in the condensation mechanism between the thiazole and pyrimidine moieties. Furthermore, growth of an *E. coli* strain (70-23) that required preformed vitamin B₁ was practically unaffected by the antagonist, whereas one mutant (70-17), which was very susceptible to the antagonist, required preformed 4-methyl-5-(β -hydroxyethyl)thiazole (in the absence of added thiamine) and, in addition, had a low ability to conjugate the thiazole and pyrimidine.

3-(α -Succinyl)-4-methyl-5-(β -hydroxyethyl)thiazole (171) could replace 4-methyl-5-(β -hydroxyethyl)thiazole in the growth of *Leuconostoc mesenteroides*.

Changes at position 5 of the thiazole greatly modified growth-promoting activity (127). Thus, acetylation of the hydroxyethyl group inhibited the growth of thiazole-requiring mutants. *E. coli* mutant 26-43 and *Neurospora crassa* 18558A [cf. (117)] responded to 4-methyl-5-(β -hydroxyethyl)thiazole for growth but not to 4,5-dimethylthiazole and only moderately to 4-methylthiazole [see below; (132)].

Only fragmentary information is available concerning biogenesis of the thiazole moiety of vitamin B₁. In view of the mechanism of thiamine monophosphate formation by yeast extracts (118 to 125), the thiazole and pyrimidine portions appear to be formed prior to condensation. In contrast, when synthesized chemically, a part of the thiazole moiety is sometimes derived from intermediates that contain groups on the carbon atom at position 5 of the pyrimidine. For example, preparations have been described (133, 134) in which 2-methyl-4-amino-5-aminomethylpyrimidine was converted to 2-methyl-4-amino-5-(N-thioformylaminomethyl)pyrimidine. This was condensed with a five-carbon precursor, such as 3-chloro-1-hydroxy-4-ketopentane, to form thiamine. Under such conditions, positions 1, 2, and 3 of the thiazole ring are derived from the thioformamide constituent of the pyrimidine. Bonner & Buchman (135) found enhanced amounts of the thiazole in pea roots in the presence of thioformamide and 3-chloro-1-hydroxy-4-ketopentane or 1-hydroxy-4-ketopentane. However, it is unlikely that 2-methyl-4-amino-5-aminomethylpyrimidine is an intermediate in the biological synthesis of thiamine in yeast, since it has been shown (122) that it is deaminated enzymically to 2-methyl-4-amino-5-hydroxymethylpyrimidine before conversion to thiamine monophosphate.

Harington & Moggridge (136) proposed that the thiazole might arise from α -amino- β -(4-methylthiazole-5-)propionic acid (Fig. 8, I), which possibly had been synthesized from methionine, acetaldehyde, and ammonia (Fig. 8, Pathway A). Their demonstration that suspensions of top yeast converted (+)- α -amino- β -(4-methylthiazole-5-)propionate to 4-methyl-5-(β -hydroxyethyl)thiazole (II) supported this proposition; however, the condensation reaction that forms the thiazole propionic acid has not been shown in biological systems. Nakayama (132) reported that *E. coli* mutant 26-43 and *Neurospora crassa* 18558 A, which can utilize 4-methyl-5-(β -hydroxyethyl)thiazole for growth, also showed a limited response to 4-methylthiazole. Furthermore, in the absence of added 4-methyl-5-(β -hydroxyethyl)thiazole, growth of both strains could be maintained by cystine or 4-thiazolidine carboxylic acid but not by homocystine, methionine, or methanol. Considerable amounts of thiamine and 4-methyl-5-(β -hydroxyethyl)thiazole were detected in cultures of the organisms that had been incubated with cystine or 4-thiazolidine carboxylic acid. As a result, a synthesis of 4-methyl-5-(β -hydroxyethyl)thiazole from cysteine was suggested involving 4-thiazolidine carboxylic acid (III) and 4-methylthiazole (IV) as intermediates (132) (Fig. 8, Pathway B). However, Korte *et al.* (137) reported that, whereas the radioactivity from 4-methyl-5-(β -hydroxyethyl)thiazole-2-C¹⁴ was incorporated into thiamine by a number of microorganisms, label could not be recovered in

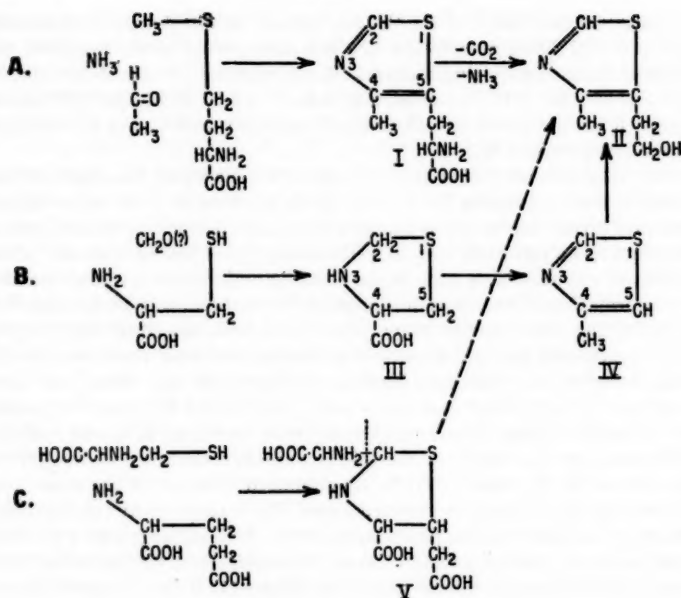


FIG. 8. Schemes of thiazole formation.

the vitamin from added 4-methylthiazole-2- C^{14} , the key intermediate in the above scheme.

Nevertheless, the demonstration by Nakayama (132) that the addition of cystine to the medium of the *E. coli* and *Neurospora* mutants promoted growth and synthesis of the thiazole moiety seems significant, and his suggestion that a thiazolidine is an intermediate in thiazole synthesis is attractive on chemical grounds. Pathway B (Fig. 8) appears not to be correct but, assuming that cysteine is indeed a structural precursor of 4-methyl-5-(β -hydroxyethyl)thiazole, alternate routes for its conversion to a thiazolidine intermediate seem possible. Thus, a precedent for the formation of such a ring from cysteine can be found in the biosynthesis of the thiazolidine portion of penicillin. In that case, the beta-carbon and the sulfur from cysteine were incorporated as a unit into positions 2 and 1 of the ring,³ respectively; the remaining atoms of the thiazolidine moiety of the antibiotic were derived intact from a molecule of valine (138). A mechanism in which the formation of the thiazole of thiamine is treated in analogous manner to the biosynthesis of the thiazolidine portion of penicillin has been outlined in Pathway C of Figure 8. Glutamic acid was selected arbitrarily as an example of how a five-carbon

³ The numbering system used here applies to thiazolidines and not to penicillin.

amino acid might condense with cysteine to form a thiazolidine precursor (V) of 4-methyl-5-(β -hydroxyethyl)thiazole.

Cocarcboxylase.—Thiamine itself, and not the monophosphate, is the direct precursor of cocarcboxylase with enzyme preparations from yeast (123, 140, 141, 150) and liver (139, 151). Studies of the distribution of the label in thiamine pyrophosphate formed from ATP³² in the presence of the purified ATP-thiamine transphosphorylase (140) have established the reaction as a pyrophosphorylation of thiamine (Equation 14). Kaziro & Shimazono (141) purified the enzyme over 100-fold from yeast extract. The preparation was free of adenylate kinase, adenine triphosphatase, nucleoside diphosphokinase, and thiamine pyrophosphatase. They reported (141) that the nucleoside triphosphates of uracil, cytosine, hypoxanthine, and guanine could replace ATP as pyrophosphate donors. Manganous ion was required as an activator. The relative effectiveness of the different nucleoside triphosphates was dependent on the concentration of Mn⁺⁺ and the pH of the reaction mixture.

A number of 1-(2-alkyl-4-amino-5-pyrimidinylmethyl)-alkylpyridinium salts showed pronounced anticoccidial activity in chickens (142). These compounds, particularly 1-(4-amino-2-*n*-propyl-5-pyrimidinylmethyl)-2-picolinium chloride hydrochloride exhibited a reversible thiamine inhibition mechanism when tested with *Eimeria tennella*, the chick, and *Lactobacillus fermentum*. The anticoccidial effect of the drugs tested was diminished upon raising the thiamine content of the diet. It is thought that the compounds may affect the thiamine phosphorylation mechanism located at the cell wall of the protozoa.

Derivatives of thiamine.—A large number of thiamine disulfides have been prepared [e.g., (143, 144)] and tested in biological systems. Some of these, such as thiamine propyldisulfide, were more effective than thiamine in promoting growth of animals, in the *in vivo* formation of cocarcboxylase, and in absorption from the intestines (145). These compounds probably do not replace thiamine but are converted to the vitamin and cocarcboxylase in the tissues. B  nhidi (146) has shown with extracts of *L. fermenti* that a reducing agent (cysteine) is required for conversion to thiamine of such compounds as thiamine disulfide and several different alkylidisulfides of thiamine. The occurrence of thiamine disulfide in yeast, first reported by Myrb  ck *et al.* (147), has been confirmed (148) in studies with vigorously aerated cultures.

The 2-trifluoromethyl analogue of thiamine has been prepared by Barone *et al.* (149) and is an antagonist of thiamine in microorganisms and mice.

MECHANISM OF ACTION

Chemical model experiments with other thiazolium compounds support Breslow's (152, 153) recent proposal for the mode of action of thiamine. Metzler and co-workers (154, 155) [see also Mizuhara & Handler (157)] have studied the chemical decarboxylation of pyruvate by thiamine and thiamine analogues. The thiazolium ring was necessary for activity. Acetoin, α -aceto-

lactate, and CO_2 , but no significant amounts of acetaldehyde, were found as products of decarboxylation. Biggs & Sykes (156) have synthesized two isomeric homologues of thiamine [Fig. 9, I and II] and found a virtually instantaneous exchange of the hydrogen atom at position 2 of the thiazolium ring with D_2O of the medium. Both compounds acted as catalysts for the conversion of pyruvate and acetaldehyde to acetoin and CO_2 (see also 157). Neither homologue showed vitamin B_1 activity in the *Kloeckera brevis* assay.

The reactivity of position 2 of the thiazolium ring has also been demonstrated in biological studies. Krampitz *et al.* (158) have presented evidence that the 2- α -hydroxyethyl derivative of thiamine pyrophosphate is probably

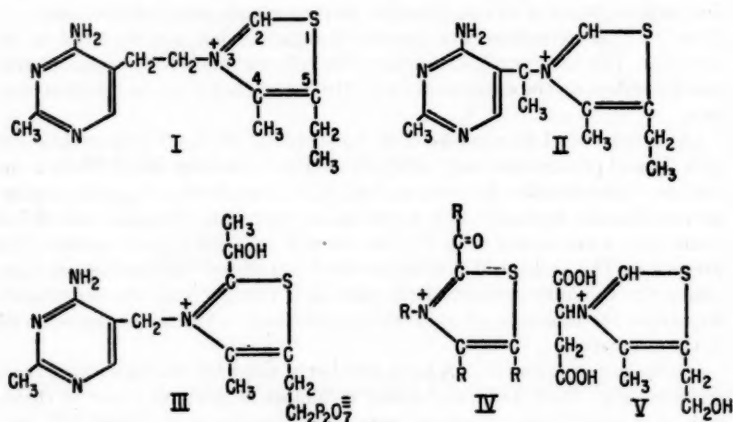


FIG. 9. Structures of thiazolium compounds.

"active acetaldehyde." Carlson & Brown (159) found that of total thiamine present in *E. coli* and bakers' yeast, 60 to 75 and 25 per cent, respectively, could be accounted for as phosphorylated α -hydroxyethyl thiamine. They also reported the formation of α -hydroxyethyl thiamine pyrophosphate [Fig. 9, III] upon incubation of cocarboxylase and wheat germ carboxylase with either pyruvate or acetaldehyde. Thiamine or thiamine monophosphate did not substitute for cocarboxylase in this system, but replacement of pyruvate with α -ketobutyrate led to a new thiamine compound, presumably α -hydroxypropylthiamine pyrophosphate. DeTar & Westheimer (160) (see also 164) showed that the hydrogen atom of the methylene bridge of cocarboxylase was not involved in biological action of the coenzyme since no exchange occurred at this group with T_2O of the medium during the decarboxylation of pyruvate with yeast carboxylase. These results were in agreement with chemical model experiments on the locus of exchange of D_2O with thiamine and its

analogues (153, 161, 162) and also eliminated the participation of the methylene group, suggested earlier as a possible active site (163), in a biological system.

Breslow & McNelis (165) and White & Ingraham (166) have observed considerable kinetic instability of 2-acyl thiazolium compounds (Fig. 9, IV), which could thus act as donors of "active acyl" groups. The behavior of these compounds is reminiscent of that of certain acyl imidazole compounds (167, 168). The properties of the 2-acyl thiazolium compounds may have a bearing on the enzymic mechanism of such cocarboxylase-catalyzed reactions as the conversion of xylulose-5-phosphate with orthophosphate to glyceraldehyde-3-phosphate and acetyl phosphate (169) and perhaps even on the elusive question of the phosphoroclastic cleavage of pyruvate (169a, 169b, 196c).

A compound containing the thiazole moiety of thiamine may be involved as a cofactor of the soluble α -glycerophosphate dehydrogenase of rabbit muscle and yeast. Van Eys *et al.* (170) had reported previously that crystalline rabbit muscle α -glycerophosphate dehydrogenase contained a non-protein component. Treatment of the enzyme with charcoal led to its removal and also to a loss of activity. Almost complete restoration of the activity of such preparations was obtained with ethylene diamine tetraacetate. Van Eys (171) reports that the properties of this component correspond to the structure of 3-(α -succinyl)-4-methyl-5-(β -hydroxyethyl)thiazole (Fig. 9, V) and that a synthetic material of this structure was able to reactivate a charcoal-treated yeast and NAD-linked α -glycerophosphate dehydrogenase of rabbit muscle. The relationship of this component to vitamin B₁ was emphasized by the demonstration that thiamine deficiency in rats resulted in a lowering of soluble α -glycerophosphate dehydrogenase.

VITAMIN B₁₂

BIOGENESIS

The disclosure of the complete chemical structure of vitamin B₁₂ in 1955 (172) focused interest on the biogenesis of two characteristic portions of the molecule, the porphyrin-like structure and the nucleotide side chain.

Porphyrin-like moiety.—Evidence that this part of the vitamin-B₁₂ molecule has a biological origin similar to that of the porphyrins came from studies with growing microorganisms by Shemin and co-workers (173, 174), who showed incorporation of radioactivity from δ -aminolevulinic-1,4-C¹⁴ into the carbon atoms of the vitamin indicated by closed circles in Figure 10. Furthermore, labeled porphobilinogen, which is a known precursor of uro-, copro-, and protoporphyrin, was also efficiently utilized in the formation of vitamin B₁₂ by certain microorganisms [Schwartz *et al.* (175)]. According to Bray & Shemin (176), six of the methyl groups (marked with asterisks in Fig. 10) arise from the methyl group of methionine. Methyl-labeled choline or betaine added to the growing cultures of the *Actinomyces* used in these studies did not contribute C¹⁴ to the C-methyl groups. Bonnett *et al.* (177) had previously suggested on chemical grounds that the methyl groups of the

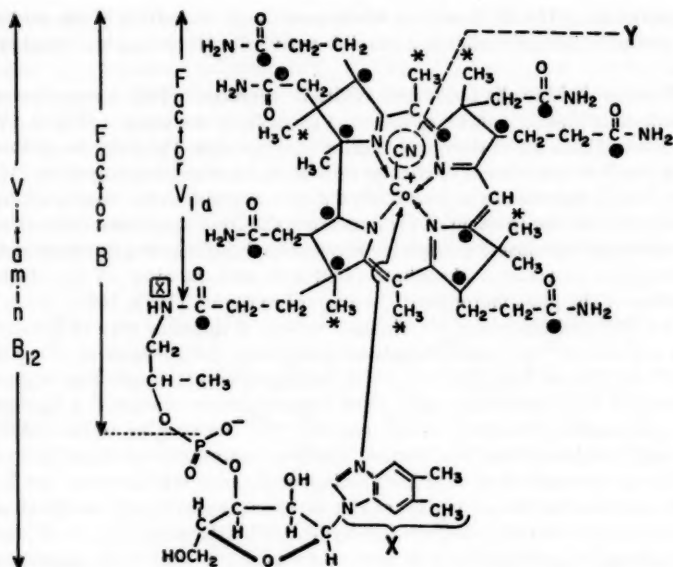


FIG. 10. Structure of vitamin B₁₂ and related compounds. Symbols used in the figure are explained in the text.

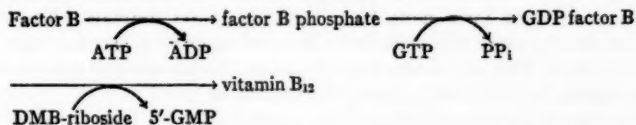
cobyrinate, now shown to come from methionine, may arise by alkylation of a partially reduced tetracyclic intermediate.

Factor V_{1a}, a monocarboxylic acid belonging to the group of incomplete cobalamines, has been isolated from sewage sludge (178), and its structure has been elucidated as cobyrinic acid abcd₆g-hexamide by Bernhauer *et al.* (179). The main proof for the chemical configuration of factor V_{1a} was its conversion to factor B (see Fig. 10) which was effected by treating a mixed anhydride from factor V_{1a} and ethyl hydrogen carbonate with 1-amino-2-propanol. Factor B phosphate was similarly prepared from factor V_{1a} (180). The complete chemical synthesis of vitamin B₁₂ from factor V_{1a}, as well as that of the analogous compound in which the phosphate group is linked to carbon 2' instead of carbon 3' of ribose, was accomplished (181). The D₆-1-amino-2-propanol moiety of vitamin B₁₂ probably arises biologically as a product of decarboxylation of threonine, since Krasna, Rosenblum & Sprinson (182) showed that in vitamin B₁₂ isolated from *Streptomyces griseus* grown in the presence of threonine-N¹⁵ the label was located almost exclusively in this portion of the molecule (position of label indicated by [X] in Fig. 10).

Nucleotide portion.—A large number of derivatives of vitamin B₁₂ are known in which the 5,6-dimethylbenzimidazole part of the molecule has been

replaced by another nitrogenous base (indicated by the symbol X in Fig. 10). Thus, pseudovitamin B₁₂ contains adenine (183) and forms of the vitamin containing a variety of other bases (2-methyladenine, 2-methylhypoxanthine, hypoxanthine, 2-methylthioadenine, guanine, 5-hydroxybenzimidazole, etc.) have been isolated from natural sources (manure, sewage, etc.). In addition, it has been possible to obtain a large number of derivatives of vitamin B₁₂ that differ in this part of the molecule by the addition of selected nitrogenous bases to the fermentation mixtures of a number of microorganisms. Excellent reviews are available which describe the chemistry, isolation, conditions of biosynthesis, and biological properties of these compounds [Johnson & Todd (172); Kon (184); Friedrich & Bernhauer (185)].

The isolation and structure of a new factor of the vitamin-B₁₂ group, quanosine diphosphate factor B, from *Nocardia rugosa* has now been described in detail by Barchielli *et al.* (186). The structure of this compound differs in certain important details from that of the cobamide derivatives described above, since it is a guanosine 5'-pyrophosphoric ester of factor B, in which ribose is linked to position N-9 of guanine. This configuration should be compared to another guanine-containing cobamide isolated from sewage residue by Friedrich & Bernhauer (187), a guanosine-3'-phosphoric ester of factor B (cf. Fig. 10) in which ribose is linked to atom N-7 of guanine. Barchielli *et al.* (186) and Boretti *et al.* (188) have proposed that quanosine diphosphate factor B and factor B monophosphate may be intermediates in the biosynthesis of vitamin B₁₂. They (188) found that radioactivity from orthophosphate-P³² incorporated into vitamin B₁₂ by *N. rugosa* was less with guanosine diphosphate (GDP) factor B or factor B monophosphate in the fermentation medium than when only factor B was present, and they have suggested the following reaction scheme for the synthesis of dimethylbenzimidazole (DMB) cobamide from factor B (186):



17.

In connection with this scheme, it may be significant to know whether the guanosine in GDP factor B is linked to ribose via an α -glycosidic bond as is the attachment of dimethylbenzimidazole in vitamin B₁₂ (189) and that of adenine at position N-7 in pseudovitamin B₁₂ (190), or whether it is a β -riboside as is the case with other ribonucleotides. It would seem reasonable that the proposed reaction of guanosine triphosphate with factor B phosphate (186) would lead to a GDP factor B containing a guanine- β -riboside bond. The enzymic mechanisms of formation of α -ribosides as they occur in vitamin B₁₂ have not been elucidated, but there is evidence that nucleosides or nucleotides containing β -glycosidic bonds may be hydrolyzed prior to utilization of the base for the formation of the vitamin B₁₂ derivatives by *E. coli* mutants [Ford & Hutner (191); Ford *et al.* (192)].

ACTION

Cobamide coenzymes.—Barker, Weissbach & Smyth (193) isolated a cofactor from *Clostridium tetanomorphum* which was required for the enzymic conversion of glutamate to β -methylaspartate. The coenzyme is a derivative of pseudovitamin B₁₂ (194, 195) (X=adenine, Fig. 10) in which the cyano group has been replaced by an adenine-containing component (see Y, Fig. 10). A benzimidazole cobamide coenzyme and a 5,6-dimethylbenzimidazole cobamide coenzyme have been obtained by growing *Cl. tetanomorphum* in the presence of benzimidazole and 5,6-dimethylbenzimidazole, respectively. The latter was also isolated from rabbit liver and a species of *Propionibacterium* (196). Other cobamide coenzymes have been synthesized in bacterial cultures from various nitrogenous bases (197). The coenzymes that contain benzimidazole and 5,6-dimethylbenzimidazole have been crystallized (198, 199).

These compounds, as in the adenyI cobamide coenzyme, differ from their corresponding vitamin-B₁₂ forms in that the cyano group is replaced by an adenine-containing material. This characteristic component was liberated from the cobamide coenzymes on exposure to light (198). Ladd *et al.* (200) have found this material to be an adenine nucleoside which yielded adenine and a reducing sugar upon mild acid hydrolysis. The latter could not be identified with any common sugar but reacted with ferricyanide, orcinol, and a number of other sugar reagents. One mole of the sugar consumed approximately three moles of periodate to yield 3 moles of formate but no formaldehyde, indicating that it may be an aldose containing three vicinal hydroxyl groups. Spectrophotometric data suggested that the sugar component was linked by a glycosidic bond to position N-9 of adenine. The adenine rather than the sugar portion of the nucleoside appears to be attached to the cobalt atom of the cobamide.

Comparative studies on the metabolism of vitamin B₁₂ and the corresponding coenzyme form have been made in intact animals (201, 202). Cyanocobalamin was more efficiently absorbed from rat intestines than the coenzyme form. The hepatic uptake of injected coenzyme was greater than that of cyanocobalamin; the reverse occurred in kidney.

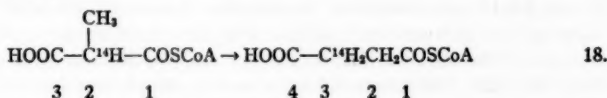
Cobamide coenzymes, as vitamin B₁₂, are bound by intrinsic factor. Ellenbogen *et al.* (203) reported that the isomerase catalyzing the conversion of glutamate to methyl aspartate was inhibited by intrinsic factor preparations. The inhibition was greater with rate-limiting concentrations of the benzimidazole cobamide coenzyme as the cofactor than with the 5,6-dimethylbenzimidazole analogue and was proportional to the concentration of added inhibitor. Intrinsic factor activity of the preparations correlated reasonably well with the degree of inhibition of the isomerase. The authors suggest that this may be a convenient *in vitro* assay for intrinsic factor.

A native cobalamin-polypeptide complex has been isolated from beef liver by Hedbom (204), yielding a single component by chromatography and electrophoresis with a molecular weight of 9100. The cobalamin content of the

polypeptide was 14.7 per cent. The infrared spectrum indicated that the cyano group was absent.

The purification of a vitamin B₁₂-active material from *Crithidia fasciculata* was reported by Sanders & Seaman (205); it is purported to differ from vitamin B₁₂.

Enzymic reactions.—Eggerer and co-workers (206) have shown that the cobamide coenzyme-dependent isomerization of methylmalonyl CoA to succinyl CoA (208 to 212) involves rearrangement of the thiolester group, since methylmalonyl-2-C¹⁴ CoA was converted to succinyl-3-C¹⁴ CoA (Equation 18) and not to succinyl-2-C¹⁴ CoA by an enzyme preparation from *Propionobacterium shermanii*.



In addition to the isomerization of methylaspartate (193 to 200) and of methylmalonyl CoA (206 to 212), the role of vitamin B₁₂ as a cofactor has been studied in other enzyme systems, especially the incorporation of amino acids into proteins and the formation of methionine and of thymidine.

Wagle, Mehta & Johnson (213, 214) reported that the addition of cyanocobalamin increased the incorporation of amino acids into protein by a cell-free preparation from livers of vitamin B₁₂-deficient rats, and they attributed this to a stimulation of the amino acid-activating enzyme (215). In contrast, Frazer & Holdsworth (216) and Arnstein & Simkin (217) did not find increased incorporation of labeled amino acids into chick or rat liver proteins by *in vitro* addition of vitamin B₁₂. Moreover, the level of the amino acid-activating enzyme appeared to be twice that of the normal in B₁₂-deficient chick liver preparations (216). Subsequently, Mehta, Wagle & Johnson (218) reinvestigated the incorporation *in vitro* of phenylalanine-3-C¹⁴ into protein and found no correlation between the activity of the amino acid-activating enzyme and the vitamin-B₁₂ nutrition of the animals. Stimulation of incorporation of labeled amino acids into protein by the addition of vitamin B₁₂ to the *in vitro* system was still reported, and the 5,6-benzimidazole cobamide coenzyme was claimed to be more effective than cyanocobalamin. Arnstein & White (219) observed that omitting vitamin B₁₂ from the medium of *Ochromonas malhamensis* did markedly reduce the incorporation of label from glucose-C¹⁴ into amino acids; but this incorporation into amino acids was much more depressed than the incorporation of amino acids into protein.

Extracts that synthesize methionine from homocysteine and one-carbon compounds have been prepared from acetone powders of *E. coli* PA 15 by Szulmaster & Woods (220). Such preparations from organisms grown in the presence of vitamin B₁₂ did not require additional vitamin in the reaction mixture for methionine formation (221, 222). However, extracts of organisms grown in the absence of the vitamin produced methionine under certain cir-

cumstances only when cyanocobalamin was added (221). Serine was usually employed as a source of the one-carbon fragment, but hydroxymethyl tetrahydrofolate could also serve as a one-carbon donor in systems that still required cobalamins for methionine synthesis; the conversion of serine to hydroxymethyl tetrahydrofolate, therefore, appears not to be under the control of vitamin-B₁₂ derivatives. Natural folic acid [a heated extract of organisms grown without vitamin B₁₂ and containing no cobalamin (223)], when used instead of tetrahydrofolate or hydroxymethyl tetrahydrofolate, replaced cobalamin with extracts from *E. coli* strain PA 15, which does not require B₁₂ for growth, but not from strain 121-176 (224), a vitamin-B₁₂ or methionine auxotroph (221, 222). Methionine synthesis by sonic extracts of *E. coli* PA 15 was stimulated by a number of cobamide derivatives (221); the reaction was competitively inhibited by the methylamide, ethylamide, and anilide of the monocarboxylic acid of vitamin B₁₂ [see also (225)] and by factor B (223). Cobalamin did not stimulate methionine formation in acetone powder extracts (222), but a heat-labile fraction from the same organism grown in the presence of cobalamin did act as a cofactor. This material has been partially purified and was found to contain cobalamin [Kisliuk & Woods (222); Kisliuk (226)]. It was suggested by Guest *et al.* (221) that the acetone treatment may have destroyed some factor involved in the metabolism of vitamin B₁₂. The cobalamin-containing fraction may well be identical with one of the three active fractions that Hatch *et al.* (227) separated from *E. coli* extract and found necessary for methionine synthesis from homocysteine and serine; the others were serine hydroxymethylase and an enzyme characteristically absent from methionineless *E. coli* [strain 205-2 (224)]. Takeyama & Buchanan (228) purified the apoenzyme of the cobalamin-containing fraction from *E. coli* 113-3 [a vitamin-B₁₂ or methionine auxotroph (224)] grown in the presence of methionine but no vitamin B₁₂. Formation of the holoenzyme from the apoenzyme and cobalamin required the presence of a sulfhydryl compound (cysteine), tetrahydrofolate, and reduced flavin-adenine dinucleotide. Reduced flavin-adenine dinucleotide was shown to be the direct hydrogen donor in methionine synthesis (229).

The 5,6-dimethylbenzimidazole cobamide coenzyme of Weissbach *et al.* (196) did not replace cyanocobalamin in the preparations of Takeyama & Buchanan (228), and it did not substitute for the cobalamin-containing fraction in Kisliuk's system (226). However, Guest *et al.* (221) have found that 5,6-dimethylbenzimidazole cobamide coenzyme was two to three times as active as cyanocobalamin in their methionine-synthesizing preparation (sonic extract) from *E. coli* PA 15, and they reported that inhibition by the anilide of cobalamin was antagonized more effectively by the cobamide coenzyme than by vitamin B₁₂ (223).

Previous experiments suggested that the *in vitro* addition of vitamin B₁₂ stimulated the conversion of formate to the methyl group of thymine in cells of *Lactobacillus leichmannii* (230) and in bone marrow from vitamin-deficient chicks (231). Cyanocobalamin was more effective than 5,6-dimethyl-

benzimidazole cobamide coenzyme in the bone marrow system (232). The locus of action of vitamin B₁₂ was narrowed to the step between "formate" and "formaldehyde" by the observation of Dinning & Young (233) that transfer of label from formaldehyde-C¹⁴ and serine-3-C¹⁴ to the methyl group of thymine was not stimulated by cyanocobalamin in bone marrow preparations. Dinning & Hogan (234) have reported that hydroxymethyl tetrahydrofolic dehydrogenase activity (see 235) from bone marrow and liver of vitamin B₁₂ deficient chicks was lower than that from normal birds. The addition *in vitro* of cyanocobalamin to bone marrow cells from deficient animals stimulated this enzyme activity, but results were erratic with the partially purified enzyme from liver.

Another class of reactions catalyzed by cobamide-linked enzymes has been added by the observation of Rabinowitz (236) that the C¹⁴O₂ pyruvate exchange of enzyme preparations from *Clostridium acidi-urici* and *Clostridium butylicum* is stimulated by a number of cobamide derivatives, especially factor B, in addition to the previously known requirements of this reaction (169b) for CoA, cocarboxylase, and orthophosphate.

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FAT-SOLUBLE VITAMINS^{1,2}

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VITAMIN A

Determination.—Spectrophotometric, colorimetric, or biological methods are used for the determination of vitamin A (1). In the two former methods, a preliminary saponification is generally required and often chromatographic purification.

If the vitamin is present exclusively in the form of all-*trans* vitamin A, it is possible as well to determine the vitamin after conversion to anhydro-vitamin A (2). However, natural sources of vitamin A generally contain several different vitamin-A isomers; moreover, isomerisation has been found to occur in products prepared from the all-*trans* vitamin (3). Since the conversion of the different vitamin-A isomers to anhydrovitamin does not give the same increase in extinction, it will be possible only in exceptional cases to apply the anhydro method without a preceding separation of the isomers (4); however, it may be used in connection with chromatographic separation for the identification of the isomers in the collected fractions (5). The transient blue color resulting from the reaction between vitamin A and antimony trichloride, which is widely used for colorimetric determination, is followed, when the antimony trichloride concentration is low, by a more stable red color, which can also be used for determination purposes (6). Chlorhydrins also produce colored reaction products with vitamin A; the reaction with glycerol chlorhydrin is the most sensitive, although those with 1-2- and 1-3-propylene chlorhydrin are most convenient because the colors produced are more stable and these obey Lambert-Beer's law (7). A colorimetric method based on the reaction with phosphotungstic acid may be mentioned (8). Also the colorimetric assay of vitamin-A preparation is complicated by the presence of isomers, since the different isomers differ in biological potency. The relative biopotency of different products may be determined, however, on the basis of the antimony trichloride method combined with maleic anhydride treatment (9).

Adsorption chromatography is generally used for chromatographic isolation of vitamin A, since it is convenient both for routine separation from other substances (10) and for the more complicated isolation of the different isomers (5). In routine separation, the losses of vitamin A are small; for instance, when using weakened alumina, the losses are of a magnitude of 2 to 3 per cent (11). Incidentally, it seems to be possible to reduce the losses by the

¹ The survey of the literature pertaining to this review was concluded in September, 1960.

² The following abbreviations are used: NADH₂ for nicotinamide-adenine dinucleotide, reduced form; FAD for flavin-adenine dinucleotide.

addition of arachis oil, which, because of its content of tocopherol, has an antioxidative action (12). Partition chromatography may also be used for the purification of vitamin A (13, 14), but this method is probably more widely used for the determination of other fat-soluble vitamins.

Particular difficulties are encountered in the determination of vitamin A in vitaminized fodder mixtures, firstly because vitamin A is generally added in small quantities and, therefore, is difficult to isolate in sufficiently pure condition, and secondly because coated vitamin-A preparations are used in which the individual particle contains such a large amount of vitamin that only a few particles will be present in the sample drawn for the analysis. Since proteins are generally used for the coating, methods that begin by extraction with light petroleum (15) cannot be used. The whole fodder sample must be subjected to saponification to ensure complete extraction (16). Collaborative examinations by this method have shown low reproducibility (17), which, statistically, has been accounted for by the above-mentioned difficulty of distribution of the coated particles in the fodder (18). The remedy must be to use larger analytical samples (19). The magnitude of the sample required is still open to discussion (19, 20).

Collaborative examinations have also shown lack of reproducibility in margarine (21). In some countries, sesame oil is added to margarine to serve as a tracer. It has been found that separation of vitamin A from the sesamin and sesamolin of sesame oil requires a special chromatographic technique (22).

The presence in various natural products of vitamin-A aldehydes, which have biological potency, calls for a method (23, 24, 25). The determination, however, is complicated by the impossibility of applying saponification, and results hitherto do, in fact, show some discrepancies. This may in part be explained by an unwanted reaction between vitamin-A aldehyde and acetone when the latter substance is used for the extraction (26).

The recognition of vitamin-A acid as a substance active in the organism (27) also calls for an analytical method. With antimony trichloride, vitamin-A acid produces an absorption band at 573 $m\mu$, but no description of the isolation and separation of this substance from other substances that react with antimony trichloride has apparently been published as yet.

Utilization.—A number of investigations on the extent to which the utilization of vitamin A depends on the route of administration seem, on the whole, to show agreement. De Man *et al.* (28) investigated the utilization of large doses and used the amount stored in the liver as the criterion. With oral administration the utilization was better in aqueous dispersions than in oily solutions. The utilization of vitamin A in aqueous dispersions was equally good with oral and intramuscular administration and somewhat better with intravenous administration. Only a small amount from an oily solution injected intramuscularly is stored in the liver. Christensen *et al.* investigated the value of large doses for the storage in the liver (29), as well as the clinical effect of smaller doses (30), and found results that agreed with those just

mentioned, except that the clinical effect is asserted to be the same in case of intramuscular and oral administration of oily solutions of vitamin A. The explanation must be that the amount of vitamin A accumulated at the site of injection is able to serve as a vitamin depot. Kring & Lund (31) have made the same observation, but they mention that the administration of a few large doses is under all circumstances less economical than smaller, daily doses, because vitamin-A destruction increases with the magnitude of the deposits. However, when the object is to transfer vitamin A from mother to offspring, intramuscular or subcutaneous administration of an oily solution is unsuitable (32). Sobel *et al.* (33) investigated the absorption of vitamin through the skin and found the utilization to be considerably lower than in the case of oral administration. When vitamin A was administered in this way, maximum utilization was obtained from aqueous emulsions.

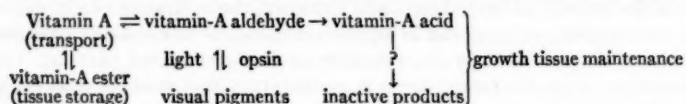
When vitamin A is mixed in feedstuffs, utilization will likewise depend on the type of vitamin preparation used. In this connection, interest has centered on the utilization of coated products, and most investigations are based on measurements of the amount stored in the liver following the administration of a large dose (34 to 38). These investigators (34 to 38) agree that in the animals examined (rats and chickens) the utilization of vitamin A in gelatin-coated products and in aqueous dispersions was equally good and considerably better than in oily solutions or products coated with fat. The composition of the feedstuff, other than the vitamin-A content, also influences the utilization. Alfalfa, for instance, contains one or more factors that improve vitamin-A absorption, and some of these factors may be obtained by aqueous extraction (39). Also the contents of vitamin E (40) and of protein (41) in the feedstuffs have a certain influence on the utilization.

The conversion of provitamins into vitamin A, which in case of oral administration normally takes place in the intestinal wall, can presumably take place in most organs and tissues (42), although this does not apply to all species of animals (42, 43). It has been possible *in vitro* to demonstrate the conversion of carotene in duodenum and in liver homogenate (44). Carotene, vitamin-A ester, β -ionone, and vitamin-A aldehyde were isolated in corresponding experiments with intestine homogenates (45). The presence of the aldehyde agrees with the scheme of Glover *et al.* for carotene conversion (46). The presence of β -apo-8'-carotenal in nature may also be mentioned as evidence of this course of the reaction, but the lower biological potency of this substance and of β -apo-8'-carotenic acid as compared with β -carotene suggests that conversion of the β -carotene molecule into two vitamin-A molecules may also take place (47). Like vitamin A, carotene is better utilized when administered in the form of aqueous dispersions than of oily solutions (49). When carotene is administered together with its natural carrier, vegetable material, the utilization will depend not only on the nature of the vegetable (50) but also on the processing of the latter. Silaging results in a considerable decrease in carotene utilization (51), which may possibly be accounted for by the destruction of the soluble carotene-protein complex

(52) in the vegetable (53). The protein content of the feedstuff also seems to influence carotene utilization (54).

When considering that the ability in animals to utilize carotene varies from species to species, it is understandable that the Vitamin Subcommittee of the International Union of Pure and Applied Chemistry concludes a report on the expression of the potency of β -carotene in terms of vitamin A by recommending that this problem be referred to the World Health Organization (55).

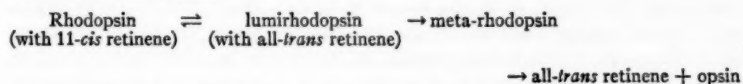
Physiological function.—Lack of vitamin A produces a number of deficiency symptoms, whose interdependency and relation to the function of the vitamin are as yet not very well-known. The action of the vitamin in the organism depends on at least two properties of the vitamin which are to some extent mutually independent. Dowling & Wald (27) confirmed the observation made by Van Dorp & Arens (56) that vitamin-A acid is able to maintain growth in rats that are fed a diet devoid of vitamin A. At the same time, they showed that vitamin-A acid is unable to ensure the formation of the visual pigments. On the basis of this, they suggest the correlation shown in the reaction between the vitamin-A derivatives and their metabolic functions:



Vitamin-A acid cannot be converted into vitamin-A aldehyde in the organism and, thus, cannot take part in the formation of the visual pigments nor result in storage of vitamin A in the liver. It is remarkable that the depletion of the vitamin store in rats fed a vitamin A-free diet is quite independent of whether or not vitamin-A acid is given. This suggests that the amount of vitamin A metabolized is independent of the extent of its function in the organism; were this not the case, there could be no agreement with the above reaction scheme or with other observations of vitamin-A losses in the organism (57). Even when very large doses were given, Dowling & Wald were unable to demonstrate the presence of vitamin-A acid in the tissues [cf. also Sharman's investigations (58)]. Following oral administration of vitamin-A acid, Sharman was unable to demonstrate the presence of this substance in the intestinal wall, but he observed an increase in the content of substances with lower absorption maxima. This seems to indicate that it is not vitamin-A acid but rather a derivative that is active in the organism. A question that now presents itself is whether it is possible to ascertain the presence of this substance in the organism in any considerable concentration.

The ability of vitamin-A acid to maintain growth in rats on a vitamin A-deficient diet made it possible for Dowling & Wald to investigate night blindness in extreme stages. They had ascertained previously that a linear relationship existed between the content of rhodopsin in the eye and the log-visual threshold (measured by electroretinography) (59). They then showed

that this relationship applied until the rhodopsin content had decreased to practically nil. Hubbard *et al.* (60) examined the conversion of rhodopsin when subjected to light, and they have presented the following reaction scheme for vertebrates:



The foregoing applies to the visual pigment of the rods, but the bleaching of the pigment (iodopsin) isolated from the cones occurs in a corresponding manner (61). The conversion of rhodopsin into lumirhodopsin has further been examined by Yoshizawa & Kito (62). A brief survey of the known importance of vitamin A to vision has been given by Pirie (63).

It is possible to maintain normal vision in rats by means of much smaller doses of 11-*cis* vitamin A than of all-*trans* vitamin A, whereas, with respect to growth, the potency of 11-*cis* vitamin A is considerably less than that of all-*trans* vitamin A (64). In this connection, it may also be mentioned that astaxanthin (65) and the corresponding 3-hydroxy-4-keto-retinene (66) maintain growth and normal vision in the animals but cannot maintain normal reproduction in either female or male rats.

Dowling & Wald's investigations of night blindness showed that vitamin-A deficiency not only resulted in destruction of rhodopsin but also of opsin, and, consequently, in partial degeneration of retinal tissue. Mention is made of the possibility that vitamin A has a similar influence on other types of tissue (59). Later investigations have ascertained that the degeneration of retinal tissue is not prevented by ingestion of vitamin-A acid (27). Whether it is also impossible to replace vitamin A by vitamin-A acid in other tissues is still open to discussion. At present it appears that further investigation of the influence of vitamin-A acid and 3-hydroxy-4-keto-retinene on the different vitamin A-deficiency symptoms might throw new light on the function of vitamin A.

Wolf and co-workers (67, 68) found that the synthesis of glycogen from acetate, lactate, and glycerol was depressed in vitamin A-deficient rats. Ingestion of cortisone re-established the power of glycconeogenesis, whereas deoxycorticosterone was without influence. It thus appears that vitamin A takes part in the biosynthesis of the cortical hormones. *In vitro* experiments, however, seem to exclude the possibility of any direct influence of vitamin A on the synthesis, although van Dyke *et al.* (48) have recently found that the conversion of cholesterol into corticosterone in adrenal homogenate from vitamin A-deficient animals was stimulated by the addition of vitamin-A acid, vitamin-A aldehyde, and vitamin-A ester. Other investigations also confirm the association of vitamin A with the steroid hormones (69, 70). Administration of progesterone, but not of pregnenolone, was able to ensure normal oestrus in vitamin A-deficient animals and resulted in an increase in the number of living newborn animals. In male rats, administration of pro-

gesterone delayed the development of vitamin A-deficiency symptoms (71). It is obvious that vitamin A influences the conversion of pregnenolone into progesterone and, presumably, several links in the biosynthesis of the steroid hormones and, consequently, the establishment of a certain balance between the latter (73). Vitamin A also seems to have a direct influence on one or more of the stages of the synthesis of cholesterol from squalene (72). However, in rats that were fed large doses of vitamin A, there was an increase in the weight of the adrenals and an increased excretion of 17-keto-steroids with the urine (74). Other investigations have also dealt with the relationship between cholesterol and vitamin A. In male, but not in female, rats it was possible to observe a reduction in the storage of vitamin A in the liver when cholesterol was given together with vitamin A, but administration of cholesterol had no influence on the vitamin A already stored in the liver (75). In humans with increased cholesterol content in the blood, a reduction has been found to result from ingestion of large quantities of vitamin A (76). It is difficult to correlate these observations with the influence of vitamin A on the cholesterol synthesis. In a great number of publications, Morton and co-workers have accounted for the significance of vitamin A to the occurrence of substance SC and substance SA in animal organs. These investigations resulted in the discovery of ubiquinone (SA) (77) and ubichromenol (SC) (78). In vitamin-A deficiency, the content of these substances in various organs will be increased. It is possible that this is caused by the reduction in the steroid synthesis resulting from vitamin-A deficiency (72).

Lack of vitamin A results in reduced basal metabolism because of reduced thyroxine secretion (79), but the administration of large doses of vitamin A also results in a reduced basal metabolism (74, 79). Farmand (80) suggests that this effect is not caused by reduction in the function of the thyroid but by the increased turnover of thyroxine in the tissues.

Vignais (81) found in vitamin-A deficiency an increase in the activity of NADPH_2^+ cytochrome-*c* reductase in liver mitochondria to about twice the normal value, whereas in thyroidectomized rats the activity of this enzyme system is reduced. Further investigations are necessary to ascertain the connection between vitamin A and the thyroid hormone.

A thorough description of the influence of vitamin A on the development of tissues has been given by Fell (82). Vitamin-A deficiency manifests itself in the development of bones through disturbances in the function of the epiphyseal plate, which results in serious skeletal deformities. It was also possible to stop the development of the bones in *in vitro* experiments in which

[†] Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-60 (November 25, 1960)], nicotinamide-adenine dinucleotide, reduced form (NADH_2) has been substituted for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH_2), for triphosphopyridine nucleotide, reduced form (TPNH).

foetal bones were incubated with large quantities of vitamin A. The matrix of cartilaginous tissue contains mucopolysaccharides, i.e., chondroitin sulphuric acid, and it has been ascertained that the presence of large quantities of vitamin A disturbed the absorption of sulphate. In experiments with pigs, Frape *et al.* (83) found increased $S^{35}O_4$ absorption in vitamin-A deficiency. With an increase in the vitamin-A supply, the absorption of $S^{35}O_4$ decreased but again increased when very high vitamin-A doses were reached. When administering overdoses of vitamin A to rats, Berdjis (84) found an increase of the parathyroid glands with histological alterations which were explained as hyperplasia. The effect of subcutaneous administration of parathyroid extract on bone formation was similar to the consequences of hypervitaminosis A. Through *in vitro* experiments with tibia from newborn rats, it was shown, however, that the effect of parathyroid hormone was less pronounced and the cartilage was not affected, whereas vitamin A resulted in almost total resorption of terminal cartilaginous tissue.

Vitamin-A deficiency results in the formation of a keratin layer in epithelial tissue, as in normal epidermis (82), whereas as a result of hypervitaminosis A the normal cornification of vagina in rats during the oestrus will not take place (85), and the normal keratinization of skin from chicken foetuses can be prevented in *in vitro* experiments (82). Redfearn & Strangeways (86) found in normal epithelial tissue only —SH groups, but in hyperkeratinized tissue they found both —SH and —SS— groups, in agreement with the theory that keratinization is accompanied by an oxidation of cysteine to cystine. Reduced cystine content in the skin of rats suffering from vitamin-A deficiency has also been observed (87). *In vitro* experiments with embryonic chicken skin by Pelc & Fell (87a) showed that vitamin A reduces the deposit of cystine and increases the DNA concentration throughout the tissue, but incorporation of tyrosine is reduced only in surface cells. In control experiments without vitamin A, keratinization was accompanied by reduced sulphate deposits in surface tissue. Incorporation of $S^{35}O_4$ into mucopolysaccharides in colon homogenates from vitamin A-deficient rats is decreased, but the ability for SO_4 -uptake is restored by vitamin A *in vitro*. The effect of vitamin A is attributable to its influence on the synthesis of 3'-phospho-adenosine-5'-phosphosulfate (91a). In goats on a vitamin A-deficient diet, keratinization of kidney epithelium with calcium deposition was observed. It is stated (88) that vitamin-A deficiency is only a predisposing factor in the formation of urinary stones.

Perhaps the importance of vitamin A for the development of tissues manifests itself most drastically through the influence of this vitamin on the development of the foetus, the viability of the young, and congenital deformities in the latter (89, 90, 91). In the discussion of the physiological functions of vitamin A, the only deformities of interest are those that may be considered to be the specific results of vitamin-A deficiency and not merely the consequences of a general disturbance of the development of the foetus. However, by far the greatest number of such congenital deformities have

also been observed following diets deficient in other accessory food factors (91).

VITAMIN D

Determination.—Chemical assay of vitamin D, which has high biological potency, is rendered difficult by the fact that it is frequently present in very low concentrations in the materials to be tested. It may be extremely difficult to isolate it in sufficiently pure condition from the large quantities of the other substances with which it occurs. In the case of products containing synthetic vitamin D, other substances formed by the irradiation will impede the determination. In this case, however, good agreement may be obtained between chromatographic-colorimetric and biological determinations (92). In the case of natural vitamin-D sources and multivitamin products, it is chiefly the presence of vitamin A that causes difficulties; this is particularly true because vitamin A will often be present in concentrations a hundred times, or more, that of the vitamin D. The presence of even a small quantity of vitamin A or of decomposition products of vitamin A in the final test solution will impede both spectrophotometric and colorimetric antimony trichloride determinations.

Vitamins A and D can be separated in several ways. By treatment with talc (magnesium polysilicate) activated by boiling with hydrochloric acid, it is possible to remove vitamin A and enable colorimetric determination (93). Chromatographic separation may be effected on superfiltrol (94), if required after a special activating process (95), on the condition that the weight ratio of vitamin A to vitamin D does not exceed 200. However, if the substances concerned are not pure synthetic vitamins, vitamin D will not be completely separated from interfering substances (95, 96), which may necessitate a correction of the colorimetric measurement. Separation may also be obtained by repeated chromatography, first on magnesium oxide-hyflo-supercel mixture and subsequently on alumina. In this case, however, a special blank determination is required to correct for the influence of interfering substances on the colorimetric measurement (the "inhibitor method") (97). Recovery values of 89 to 106 per cent of added vitamin D were obtained by applying this method to fortified evaporated milk (98).

The difference in the solubilities of vitamins A and D allows for separation by partition chromatography. Polyethylene powder with a butanol-methanol-water mixture as the mobile phase (99) or Celite impregnated with polyethylene glycol 600 with iso-octane as the mobile phase (100) are examples. Separation may also be made by modifications of the paper chromatographic method (101, 102). All in all, at the moment a combination of partition chromatography and adsorption chromatography may be considered to be the most convenient for the isolation of vitamin D. Even so, it is hardly possible in the case of low-potency vitamin-D preparations to purify the material sufficiently to permit direct spectrophotometric or

colorimetric measurement. This is indicated by the necessity of using the inhibitor method. The application of gas chromatography for microanalytical separation of steroids (103) opens the possibility of using gas chromatography for vitamin-D assay. According to the chromatographic methods hitherto applied, it has not been possible to separate vitamin D₂ and vitamin D₃, but it is possible to distinguish between them by a color reaction with furfural and sulphuric acid (104).

It is difficult to obtain sufficiently reliable results from chemical methods, and biological methods are still used as official assay methods (106).⁴ There is a tendency to attach less importance to the actual procedure as the requirements for reproducibility and accuracy are raised (105). Proposals for alternative details in established official methods (107, 108) show the same trend. For a biological determination of materials with a low vitamin-D potency, preliminary saponification and extraction are often required (109).

Utilization.—The utilization of vitamin D has been investigated—as has that of vitamin A—by studying the different routes of administration. Oral administration of relatively small quantities, whether in aqueous dispersion or in oily solution, provided equally effective protection against rachitis. On intramuscular administration, an aqueous dispersion gave somewhat higher results (110) than oily solutions, where a very slow absorption was observed. This had a good prophylactic effect but was only slightly curative. Previously, intramuscular administration of large doses of vitamin D in oil had been found to have only small curative effect (111). Accumulations of the vitamin at the site of injection may serve as a depot for up to 200 days, but they are only slowly absorbed. Small daily doses given orally are generally more effective than large doses given at intervals (112, 113). The utilization of coated vitamin-D products is only slightly lower than the utilization of vitamin D dissolved in alcohol (114). In a series of investigations, Kodicek and co-workers have accounted for the absorption and storage of vitamin D following oral and intramuscular administration [Kodicek (115)]. When vitamin D is given orally in doses from 400 to 160,000 I.U. to normal, as well as rachitic, rats, it is possible to collect from the feces quantities of vitamin D and its decomposition products corresponding to 70 per cent of the intake. Of the 30 per cent retained in the organism, only one-third, i.e., 10 per cent of the intake, will be present as vitamin D, chiefly in the liver. The utilization is equally low whether the vitamin D administered is dissolved in oil or in water-Tween 80. The absorption of vitamin D takes place in the lower half of the small intestine. Blumberg *et al.* (116) found similar distribution of orally administered vitamin D. However, rachitic rats absorbed somewhat more than non-rachitic rats. The absorption process must be different in man, since no vitamin D can be found in the feces fol-

⁴ An exception to this appears in the *U. S. Pharmacopoeia*, XVI (pp. 910-12) recently issued, in which the biological method hitherto prescribed has been abandoned in favour of the chemical inhibitor method (97) already mentioned.

lowing oral intake of a considerable quantity (116). After intramuscular administration to rats, only a small percentage will be excreted as vitamin D in the feces, but the quantity accumulated in intestinal tissue is as large as following oral administration (115). It is possible that the stimulation of calcium absorption by vitamin D is dependent on the presence of the vitamin in intestinal tissue. Raoul & Gounelle (117) found that vitamin D given by intramuscular injection could be collected almost quantitatively from the adrenals 30 min. after the injection. After that period it was gradually released to other organs. Kodicek *et al.* (118) were unable to verify this observation.

Synthesis on irradiation.—Vitamin-D synthesis on ultraviolet irradiation of skin is of the magnitude of 30 to 100 I.U. vitamin D per sq. cm. of pig (119); with human skin, 4 to 18 I.U. per sq. cm. have been found (120). In rats, the value is 5 to 15 I.U. per sq. cm., which corresponds to 15 to 30 per cent of the provitamin D present (119). It amounts to 290 I.U. for the whole area of the skin when the fur has been removed prior to irradiation (121).

Physiological function.—The importance of vitamin D in calcium absorption may be demonstrated by means of everted sacs of the small intestine. Such sacs, when taken from rachitic rats, show only half of the calcium absorption of sacs from rats that have been given small doses of vitamin D (122). Calcium and phosphate deposition in the bones is associated with the influence exerted by vitamin D on their concentrations in the blood; these are lower in rachitis (123). Harrison *et al.* (124), studying rats that were given a diet with sufficient calcium and phosphate but no vitamin D, found that the content of calcium in the blood was low but increased when vitamin D was added to the diet. Also, the phosphate content increased when vitamin D was administered, but only for a brief period, dropping again to the original value. In rats that were given very little calcium, administration of vitamin D resulted in a considerable increase in the serum concentration of calcium but in no significant increase in the absorption of calcium (125). The action of the vitamin, therefore, must be to mobilize the calcium of the bones. Several investigations have demonstrated the importance of the calcium-phosphorus product in the blood to the deposition in the bones (126, 127, 128). It is natural to correlate the results of vitamin-D deficiency with the activity of the parathyroid glands, the influence of which on the calcium content of the blood is essential. In normal rats, the hormone will result in an increase of the serum calcium, but in rachitic rats it cannot produce a corresponding effect (124). On the other hand, the absence of the parathyroid hormone did not impair the response of the calcium absorption to the administration of vitamin D (129).

The importance of vitamin D to the deposition of calcium and phosphate in the bones is not only a question of the concentration of these substances in the blood; there is no doubt that vitamin D is also necessary to enable cartilaginous tissue to incorporate calcium and phosphate. In normal cartilagi-

nous tissue a substance has been found which is not present in corresponding tissue from rachitic rats, but which is formed when vitamin D is administered (131). The ability to mineralize cartilaginous tissue is only recovered, however, about two days after the administration of vitamin D (132). At the same time, the matrix gives off large quantities of a substance that shows an ability to bind calcium *in vitro*, possibly chondroitin sulphate. Reduced ability to synthesize glucosamine from glutamine and glucose-6-phosphate is found in cartilaginous tissue from rachitic rats (133). *In vivo*, however, a drop in the content of hexosamine in the cartilaginous tissue is observed when vitamin D is given to rachitic rats [compare the above observation with respect to the chondroitin sulphate given off by the matrix]. The difference in the epiphyseal cartilage in rachitic and non-rachitic rats may also be demonstrated by a special staining technique (134). It is reasonable to assume that the influence of vitamin D on the calcium absorption may be explained by the fact that the vitamin affects the ability of the intestinal tissue to absorb and transfer calcium.

The effect of vitamin D on bone is differential insofar as the addition of this vitamin impedes development of certain parts of the skeleton, while furthering the development of others without altering the relative contents of minerals (125). The content of alkaline phosphatase in the blood, which is increased in rachitis, is assumed to be of importance in the deposition of calcium and phosphate. However, Gran & Eeg Larsen (135) were unable to confirm the theory that vitamin D stimulates the activity of alkaline phosphatase. It had previously been ascertained that vitamin D increased the citrate concentration in certain tissues and in the urine and lessened the conversion of citric acid to α -keto-glutaric acid. In agreement with this, tibial cartilaginous tissue from rachitic rats on incubation with citrate showed reduction of the oxygen consumption and increase of the α -keto-glutarate concentration (136). The difference between rachitic and non-rachitic groups in this case was in the phosphate intake and not in the vitamin-D intake. On the whole, many experiments on rachitis have been based on rats fed an experimental diet which, for the purpose of producing a rachitic condition, was not only deficient in vitamin D, but also in calcium and phosphorus. Such experiments are not immediately relevant to the function of vitamin D. In epiphyseal cartilage from rachitic rats, Meyer *et al.* (137) found reduction not only of the citrate oxidation but also of the citrate synthesis. By incubation of palatal mucosa with succinate or malate, Zar & Irving (138) found an increase in the oxygen consumption after addition of phosphorylated vitamin D, i.e., a somewhat different influence on the citric acid cycle than that demonstrated in the case of cartilaginous tissue. Incidentally, it is not very probable that this influence is the result of direct action of vitamin D on the enzyme system in the citric acid cycle. By means of cortisol it is possible to prevent this action of vitamin D without influencing the anti-rachitic effect (139).

VITAMIN E

Chemistry, analysis, and occurrence.—Continued investigations of the natural tocopherols have shown that we should now add to the list of methyl-substituted tocols a new series of substances which differ from the tocol derivatives by having three double bonds in the C-2 side chain (140, 141). Two substances of this series have been found to date. Thus, natural ϵ -tocopherol, which may be isolated from wheat bran, is not, as hitherto assumed, identical with 5-methyl tocol but is an unsaturated analogue of β -tocopherol (140). ζ -Tocopherol, which occurs in palm oil and wheat bran, is similarly an unsaturated analogue of α -tocopherol (5,7,8-trimethyl tocol), whereas the ξ -tocopherol (ξ_2), which occurs in rice, is apparently, as originally assumed, identical with 5,7-dimethyl tocol (141). Final proof of the structure of these substances in the form of a total synthesis is still lacking.

The interest in paper-chromatographic methods of tocopherol analysis is still considerable. The Society for Analytical Chemistry has published a detailed description of such a method on the basis of the work performed by The Vitamin E Panel (142). The method is based on two-dimensional paper chromatography and includes such preliminary steps as extraction, saponification, separation of sterols by freezing from methanol solution, and purification on flordin earth. This seems to be the most convenient method for determination of the individual tocopherols, but the great number of precautions required would appear to render the method less suitable for routine quantitative analysis. This method, or modifications of it, has been used for the determination of tocopherols in margarine (143), oats (144), and animal tissue (145). Green (146) investigated the tocopherol pattern in young, growing plants of maize, wheat, barley, and pea and found mostly α -tocopherol during the period of vigorous growth and cellular differentiation, which suggests that a conversion of the non- α -tocopherols of the seed in the resting stage to α -tocopherol may take place during the development of the plants.

Complete separation of the known tocols is not possible by the conventional two-dimensional paper-chromatographic method, since β - and ϵ -tocopherol do not separate from γ - and η -tocopherol, respectively. However, this problem has been solved by Marcinkiewicz & Green (147), who describe a complete separation and determination of β -, γ -, δ -, ϵ -, and η -tocopherols by paper chromatography of the nitroso derivatives. Separation on the basis of a coupling reaction between tocopherols with free 5-position and diazotized dianisidine was not feasible (148).

A spectrophotofluorometric method for the determination of vitamin E (149) and a new color reaction with crystals of 2-hydroxy-3-methyl-1,4-naphthoquinone and cysteine hydrochloride (150) have been published. Descriptions are also given of modifications of some of the conventional methods for vitamin-E determination in plasma (151), milk (152), and grass (153) and for the determination of free α -tocopherol in oil seeds (154). In

whole wheat, 80 per cent of the total vitamin-E content has been found in the ground outer endosperm (155), although the germ is generally assumed to have the greatest content of this vitamin.

Bioassay and biopotency.—Friedman *et al.* (156) have modified and standardized the rapid and simple method of bioassay for vitamin E which depends upon the *in vivo* action on dialuric acid-induced hemolysis of rat erythrocytes. The relative activities of the racemic and optically active forms of α - and γ -tocopherols and esters of these tocopherols have been examined, and they are in close agreement with the activities determined by the officially accepted rat anti-sterility tests (157). The same method has been used by Sharman & Richards (158) for the examination of the vitamin-E potency of bread prepared from flour bleached with chlorine dioxide and of bread enriched with wheat germ. The results agree well with those found by chemical determination of tocopherol contents in bread.

Green *et al.* (159) examined the relative biological activities of the known tocopherols and of the two above-mentioned natural ϵ - and ζ -tocopherols. The examination of the substances was based either on the effect on the respiratory decline in necrotic rat liver following intraportal administration (159) or on the *in vivo* and *in vitro* effect on hemolysis (160). The *in vitro* effect of the individual tocopherols showed considerably smaller differences than the *in vivo* effect, a fact which suggests that the biological specificity is partially lost in the *in vitro* experiments. On the other hand, the results show fairly good agreement between the activity measured after intraportal injection and after oral administration, which indicates that the biological selectivity that determines the relative activities of the tocopherols is not solely dependent upon the intestinal walls, as generally assumed.

The curative effect of intravenous administration of α -tocopherolhydroquinone on muscular dystrophy has been re-examined and confirmed (161). The disuccinate of the hydroquinone had a very small effect, however, because of either slow hydrolysis of the ester or rapid excretion or metabolism.

The enzymatic role of vitamin E.—The role of α -tocopherol in the electron transport system has evoked considerable interest. Several reviews on this topic have been published; in particular, mention should be made of those of Slater (162) and Nason (163, 164).

The theory that tocopherol functions as a cofactor in the cytochrome-c reductase system in the respiratory chain, as suggested by Nason and co-workers, apparently cannot obtain sufficient experimental support. Attempts to restore the activity in iso-octane-extracted enzyme preparations show that the reactivation does not show specific dependency on α -tocopherol. Weber and co-workers (165, 166, 167), Marinetti *et al.* (168), and others (169, 170, 171) found that a great number of substances, chiefly of lipid nature, were active; for example, vitamin K, ubiquinone, phytol, neutral fat, Tween 80, and certain hydrocarbons. Pollard & Bieri (172) have further shown that physical processes such as freeze-drying, lyophilization, and centrifuging

restore the activity in the iso-octane-extracted enzymatic preparations. These results, together with a direct demonstration of the inhibiting effect of iso-octane and other hydrocarbons (167, 172), suggest that the inactivation is an experimental artifact. Thus, residual iso-octane adsorbed on the surface of the enzyme can cause the observed inhibition (173).

Subsequently, the Nason group (174, 175, 176) described experiments with aged cytochrome-*c*-reductase preparations with or without iso-octane extraction and found that α -tocopherol had a specific reactivating effect, whereas tocopheryl acetate, tocopheryl quinone, and other lipids, which were active in the solvent-inhibited preparations, had no effect in this case. Pollard & Bieri (177), in duplicating these experiments, found, on the contrary, that not only tocopherol but also certain antioxidants and phenolic compounds were able to reactivate cytochrome-*c*-reductase preparations after ageing, and they postulated that the activity-depressing effect of ageing was caused by a formation of lipid-peroxides.

Another essential point in connection with the possible enzymatic role of tocopherol is the question of whether tocopherol metabolites are present in the enzyme preparations. Bouman & Slater (178) and Donaldson & Nason (179) have submitted results which indicate that tocopherol in certain tissues is present chiefly as tocopheryl quinone. Slater (162), however, emphasized that the quinone, which following acid reduction is determined as α -tocopherol, might be ubiquinone, and this later proved to be the case (180). Martius & Costelli (181) stated that, following administration of labelled α -tocopherol to rabbits, they could isolate from the liver mitochondria a metabolite which on acid reduction yielded α -tocopherol but which resembled trimethylphytylbenzoquinone rather than α -tocopherylquinone. It does not appear probable that the metabolite in this case could be identical with ubiquinone, since Morton & Phillips (182) and Moore (183) have shown that neither intake nor deficiency of vitamin E has any influence on the ubiquinone concentration in the tissues and Alaupovic & Johnson (184), after administration of C^{14} -labelled D- α -tocopherylsuccinate, were able to isolate only non-radioactive ubiquinone. Consequently, it does not seem possible at the moment to ascribe to tocopherol per se a role as electron carrier.

Schwarz (185) has drawn attention to a new class of tocopherol derivatives with benzoquinone structure which had previously been isolated by Simon *et al.* (186) from urine by means of C^{14} -labelled tocopherol. By means of liver-slice preparations in a Warburg substrate, they showed that *in vitro* addition of the Simon metabolite prevented the respiratory decline observed in necrotic livers from rats on a *Torula* yeast diet deficient in vitamin E and factor 3 (187). α -Tocopherol had no effect in these experiments and could only prevent the respiratory decline when added to the diet or given intravenously before the extirpation of the liver. In liver homogenates from necrotic animals suffering from deficiency symptoms, no decrease in the oxidation was observed when glucose was the substrate and ATP and diphosphoryridine

nucleotide were added (188). On the other hand, Corwin & Schwarz (189) have recently described another homogenate with α -keto-glutarate or succinate as substrate; it showed a decrease in oxidation which could be prevented by *in vitro* addition of α -tocopherol, the tocopherol metabolite, diphenyl-*p*-phenylenediamine, menadione, and methylene blue.

Biochemical alterations in vitamin-E deficiency.—There was no change in the NADH₂ cytochrome-*c*-reductase activity in liver mitochondria from rats fed a vitamin E-deficient diet (190). Increased succinic acid-cytochrome-*c*-reductase activity may be observed, however (191), particularly during the last part of the deficiency condition (192). The mitochondrial stability is concurrently lessened, and Corwin & Lipsett (191) assume that the apparent increase in the activity is due to increased accessibility to the substrate for the enzyme. This is in agreement with the results found by Zalkin & Tappel (193), who demonstrated increased *in vivo* lipid peroxidation in liver mitochondria from vitamin E-deficient rabbits; they are of the opinion that tocopherol stabilizes the content of unsaturated lipids in the cells against oxidative destruction and thereby maintains the structural integrity at the subcellular level.

Investigations of the creatine metabolism in vitamin-E deficiency have been continued. The creatinuria and the low content of creatine in the muscles of monkeys suffering from vitamin-E deficiency is accompanied by an increased content of DNA and RNA in bone marrow and of DNA in muscles (194). By examination of liver homogenates, the synthesis of creatine from glycocyamine and methionine was found to be normal (195). Fitch & Dinning (196) gave vitamin E-deficient rabbits and rats supplements of P₃, phosphate and found an increased turnover of intracellular inorganic phosphate in skeletal muscles, but the results did not show clearly whether the phosphorylation of creatine and ATP was reduced or normal. Other investigations, however, seem to show that the phosphorylation of creatine is reduced. Carpenter *et al.* (197) have shown that the transfer of phosphate from creatine phosphate to glucose-1-phosphate is reduced in vitamin-E deficiency in spite of normal glycolysis and phosphoglucomutase activity. Moreover, Nesheim *et al.* (198) have found that the contents of active and total phosphorylase and glycogen in striated white breast muscles are reduced in chicks fed a vitamin E-free *Torula* yeast diet.

Glycine in some way seems to play a role in the dystrophic condition which results from vitamin-E deficiency (199). Tallan (200) had previously shown that in muscle extracts from dystrophic rabbits the contents of most of the free amino acids are unchanged, whereas the glycine content is greatly reduced. Smith & Nehoyaran (199) found reduced phosphatase activity in vitamin E-deficient rabbits, which could be prevented by a supplement of 1 per cent glycine to the diet. This deficiency condition is characterized by considerable amino aciduria (201, 202) in which the glycine content in the urine is not increased (202). The most significant increase in the content of

free amino acids in the urine is observed in the case of 1-methyl histidine, which represents 75 per cent of the total amino acid content and which, incidentally, is the earliest sign of the deficiency, occurring even earlier than the creatinuria (203). Moreover, glycine, anserine, and carnosine are also present in diminished quantities in dystrophic muscles, presumably because a reduction of the synthesis of the substances occurs (204). However, the decrease in anserine content is neither rapid enough nor sufficiently large to account for the increased excretion of methyl histidine (203).

Antioxidant relationships.—The relative antioxidant activities of the known tocopherols have been investigated by Lea & Ward (205). As an empirical rule, activity decreases in the order: monomethyl tocol, dimethyl tocol, trimethyl tocol; and in the order: 8-methyl tocopherol, 7-methyl tocopherol, 5-methyl tocopherol. There are, however, a few exceptions.

Investigations of the vitamin-E activity of chemical antioxidants have been continued. Ethoxyquin (Santoquin; 1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline) prevents both encephalomalacia and exudative diathesis as well as the *in vivo* peroxidation of liver lipids in chicks on vitamin E-free diet (206). If the effect of the antioxidant in this experiment is the result of a protection of the vitamin E in the body, the vitamin-E requirements must be extremely low. Using diphenyl-*p*-phenylenediamine, Draper *et al.* (207) found not only a preventive but also a curative effect on a severe vitamin-E deficiency as measured by the fetal resorption in rats. This suggests that it is a direct biological effect, not caused by vitamin-E sparing.

Contrary to these results, Shull *et al.* (208) could not prevent muscular dystrophy in vitamin E-deficient guinea pigs by means of diphenyl-*p*-phenylenediamine, ethoxyquin, butylated hydroxy-toluene, or 2,5-di-*t*-butyl hydroquinone; nor could they prevent an increase in the cholesterol content in plasma and skeletal muscles. Methylene blue prevented the brown discoloration of uterus and degeneration of testes in rats on vitamin E-free diet, but it had no influence on the hemolysis of erythrocytes in the dialuric acid test (209).

An antagonistic action between tocopherol and unsaturated fatty acids may be observed. This appears to explain the fact that cod liver oil cannot prevent symptoms of vitamin-E deficiency, although it contains 100 μ g. α -tocopherol per gm., which is four to 12 times greater than the vitamin-E requirement in a diet containing lard instead of cod liver oil (210). Dam *et al.* (211, 212) found that an addition of polyunsaturated fatty acids to a vitamin E-free diet causes a considerable aggravation of exudative diathesis and encephalomalacia in chicks. A supplement of various fatty acids to a fat-free bakers' yeast diet gave increased frequency of liver necrosis in rats, the frequency increasing with the degree of unsaturation (213). With linolenic acid as supplement, liver necrosis was observed in all of the animals, and it is suggested that in vitamin-E deficiency autooxidation of unsaturated lipids may take place in the tissues and that this is responsible for the lesions of the

tissues. Bieri *et al.* (214) are of the opinion that the anti-vitamin-E activity of *Torula* yeast, besides depending on the mineral substances, is also caused by the content of unsaturated fatty acids in this yeast. This agrees well with results found by Tappel & Zalkin (215, 216), who demonstrated *in vivo* peroxidation in the liver of vitamin E-deficient rabbits and increased lability of isolated liver mitochondria measured by the NADH₂ cytochrome-*c*-reductase activity. It is assumed that vitamin E primarily acts as an antioxidant and thereby protects the structural integrity and enzymatic activity of the mitochondria.

Vitamin E and selenium.—This subject has been dealt with in detail by Schultze in Volume 29 (1960) of the *Annual Review of Biochemistry* and has therefore been omitted from the present review.

VITAMIN K

Analysis, chemistry, and biological activity.—A new color reaction with xanthane hydride has been proposed by Schilling & Dam (217) and has been used for the determination of vitamin K₁ in alfalfa after chromatography on secondary calcium phosphate (218). For products with lower concentrations and high contents of interfering substances, e.g., related benzoquinones (219), the purification involved is hardly sufficient. Lev (220, 221) has examined the vitamin-K requirements of rumen strains of *Fusiformis nigrescens* and has thereby opened up the possibility of a microbiological assay method; however, the technique is not yet suitable for routine work.

An excellent survey of the chemistry and biochemistry of vitamin K has been published by the Isler group (222). By total synthesis and comparison with natural vitamin K₂ isolated from putrefied fish meal, the structure of vitamin K₂ has been established as 2-methyl-3(all-*trans*-farnesylgeranylgeranyl)-1,4-naphthoquinone; i.e., at position 3 there is a side chain with 35 carbon atoms consisting of seven isoprene units (223). According to Isler's nomenclature, the substance is denoted as vitamin K₂₍₃₅₎. In addition, a number of other vitamin-K₂ compounds were synthesized, having side chains with 10, 15, 20, 25, and 30 carbon atoms, respectively. Several of these substances occur in nature. In putrefied fish meal, Isler *et al.* (223) found also small amounts of vitamin K₂₍₃₀₎; i.e., the substance that Doisy had assumed to be the natural vitamin K₂. Kruse Jacobsen & Dam (224) isolated from various bacterial strains three different types of vitamin K, among which was vitamin K₂₍₃₂₎. The vitamin K-active naphthoquinone isolated by Brodie *et al.* (225) from *Mycobacterium phlei* could not, however, be identified as belonging to the vitamin-K₂ series.

Natural vitamin K₁ with 20 carbon atoms in the side chain shows greater biological activity than analogues with either more or fewer carbon atoms (226). On the other hand, in the vitamin K₂ series the natural vitamin K₂₍₃₅₎ was less active than analogues with 20, 25, and 30 carbon atoms; K₂₍₂₅₎ showed the highest activity. Geometrical isomers of vitamin K₂₍₃₀₎

and $K_{1(20)}$ were likewise tested on vitamin K-deficient chicks 6',7'-Mono-*cis* isomers in which the *cis* double bond is near the naphthoquinone ring were considerably less active than, for example, the 10',11'-mono-*cis* compounds or the all-*trans* isomers. Martius & Esser (227) administered C^{14} -labelled menadione to chicks and rats and isolated radioactive vitamin K, which in countercurrent distribution of the bis-(*p*-bromobenzoyl) derivative behaved as vitamin $K_{2(20)}$. The authors consider it probable that all vitamin K-forms after oral administration are converted into menadione and then add the 20-C atom chain.

Comparisons between the biological activities of vitamin K_1 and menadione have been performed by means of the prothrombin test applied to

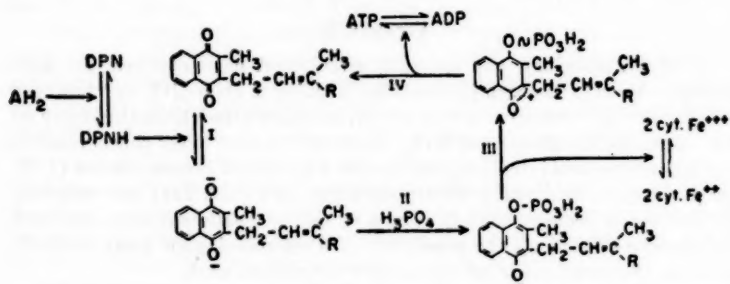


FIG. 1. Proposed mechanism for the action of a naphthoquinone monophosphate in oxidative phosphorylation (235).⁶

chicks (228, 229, 230). The sodium bisulphite compound of menadione was found to be 1.5 times as potent as vitamin K_1 (230).

The enzymatic role of vitamin K.—Several articles surveying the literature on the enzymatic role of vitamin K, especially in oxidative phosphorylation, have been published [see Martius (231, 232); Dam & S ndergaard (233)].

Harrison (234) and Brodie & Ballantine (235) have formulated a probable reaction scheme (Fig. 1) of the oxidative phosphorylation; according to this scheme, the naphthoquinone has two functions: as electron carrier between two coenzymes in the respiratory chain and as catalyst for the generation of energy-rich phosphate bonds. In the first stage of the reaction, the naphthoquinone is reduced to hydroquinone by the transfer of electrons from $NADH_2$, presumably by an FAD-linked enzyme (236) corresponding to the vitamin-K reductases isolated by Martius & M rki (237) and Wosilait

⁶ According to a personal communication from Arnold F. Brodie, recent investigations indicate that chromanol phosphates and not quinol phosphates as stated in the scheme are intermediates. A ring closure takes place between the side chain at position 3 and the oxygen atom at position 4.

(238) from calf and dog liver, which are inhibited by dicumarol. The hydroquinone is phosphorylated and then oxidized, with cytochrome as the electron acceptor while giving off energy-rich phosphate to ADP, probably with a highly unstable compound, the enol phosphate ester, as an intermediate.⁸ In a model compound experiment using a monophosphorylated naphthoquinone (mono-ethyl menadiol monophosphate) and with ADP as the only phosphate acceptor, Brodie & Davis (239) demonstrated the formation of ATP. This required the addition of both particulate and supernatant fractions from a cell-free *Mycobacterium phlei* extract. The particulate fraction contained dehydrogenases, cytochromes-*c*, -*b*, and -*a*. Kinetic studies indicate that the supernatant fraction contains at least two protein components, one for oxidation and one for phosphorylation (240).

Irradiation of bacterial extracts with light at 360 m μ destroys a vitamin K-like substance which is necessary for oxidative phosphorylation without destruction of the apoenzymes (235, 241, 242). Addition of vitamin K or closely related homologues (241, 242, 243) is specific for the reactivation of both oxidation and phosphorylation. Reactivation of the oxidation alone may be effected with riboflavin phosphate, FAD (241), or naphthoquinones substituted in either position 2 or 3, e.g., lapachol or menadione (235). With succinate as substrate, an unknown component in the supernatant liquid is required for the reactivation (241).

Reduced menadione is rapidly oxidized in the presence of respiratory chain preparations according to a first-order reaction scheme (244). The reaction is inhibited by cyanide (244, 245). Also cyclic photophosphorylation is catalyzed by vitamin K, presumably with an alternative pathway catalyzed by flavin mononucleotide (246). The latter pathway is dependent on the presence of NADP and is more sensitive to inhibition with dinitrophenol and *o*-phenanthroline than the former. Both pathways are inhibited by *p*-chloromercuribenzoate but not by antimycin-A. The oxidative phosphorylation of glutamate and succinate in liver mitochondria from vitamin K-deficient chicks could not be correlated with the prothrombin time in experiments by Beyer & Kennison (247); this is contrary to previous results of Martius & Nitz-Litzow (248).

Vitamin-K requirements.—Investigations of vitamin-K requirements are being continued. In rats on vitamin K-free diet and prevented from coprophagy, the prothrombin time increases but becomes normal when 1 μ g. menadione or vitamin K₁ per 100 gm. of body weight is administered (249). Mameesh & Johnson (250) found that 5 μ g. per rat per day was sufficient to prevent hemorrhage and prolonged prothrombin time, irrespective of whether the animals were coprophagous. In the case of turkeys on a vitamin K-free diet, 0.8 mg. menadione per lb. feed or a single oral dose of 6 to 8 μ g. per 100 gm. body weight was required to maintain normal prothrombin time (251).

Addition of substances such as sulphaquinoxaline, nicarbazin, arsanilic acid, aureomycin, or arsonic acid to a vitamin K-free diet administered to

chicks prolonged the clotting time and caused hemorrhage (252). Menadione counteracted the increase in the prothrombin time but did not influence the hemorrhagic condition. In experiments with chicks on a vitamin K-free diet, Griminger (253) found that when a single dose of about 50 mg. sulphaquinoxaline was given orally with varying doses of menadione, one mole of menadione was sufficient to counteract the inhibiting effect of more than 800 moles of sulphaquinoxaline. Chicks with coecal coccidiosis are assumed to have an increased vitamin K requirement (254). The magnitude of this increase in the requirements has not been finally determined (255).

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THE BIOCHEMISTRY OF MUSCLE^{1,2}

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INTRODUCTION

During the period covered by this review, many more papers of significance to the field of muscle biochemistry have been published than can be usefully mentioned in an article of this size. Discussion therefore has been confined mainly to those fields in which important developments are occurring. Aspects of metabolism that are not strictly unique to the muscle cell but are of significance for the biochemistry of other tissues have been omitted in the hope that they will receive mention elsewhere in this volume. Books on muscle which have appeared during the last three years include Weber's Dunham lectures (1) and the first volumes of a treatise on muscle (2); the latter promises to be the authoritative work on this subject and contains several articles specifically concerned with biochemical topics (3, 4, 5). Reviews on various aspects are also available (6 to 8a), as are the papers presented to several recent conferences devoted to muscle (9, 10, 11). The comparative aspects of muscular contraction have received some attention in general reviews (12, 13) and in an article on insect flight muscles by Boettiger (14).

NON-CONTRACTILE SYSTEMS

Sarcoplasm.—The electrophoretic pattern of the myogen fraction of bovine muscle is comparable to that of rabbit muscle, although some variation from muscle to muscle and animal to animal has been reported (15). On the other hand, myogen extracts of carp and plaice are similar, but they are electrophoretically different from rabbit (16). Although apparently not present in the rabbit, several crystalline proteins, namely myogen I, myogen II, and a low molecular weight protein of unusual composition (17, 18), can be obtained from both fish. Rabbit sarcoplasmic proteins differ from the proteins of the soluble fraction of liver and brain in that most of them are eluted from diethylaminoethyl cellulose at low ionic strength (19, 20). When the activity of rabbits is restricted, the total nitrogen content of the skeletal muscle remains unchanged, but the sarcoplasmic protein increases at the expense of the myofibrillar fraction (21, 22). A feature of some molluscan muscles is that extracts made at low ionic strengths exhibit streaming birefringence attributable to the extraction of fibrous proteins of the contractile system (23, 24). The observation (25, 26) that the proteolytic activity of

¹ The survey of the literature pertaining to this review covers the period from October, 1957, to September, 1960.

² The following abbreviations are used: ATPase for adenosine triphosphatase; DNP for dinitrophenol; ITP for inosine triphosphate.

aqueous extracts of rat muscle has a high pH optimum may explain earlier reports of low proteolytic activity in this issue.

Granular components of sarcoplasm.—The numerous studies that have been reported on muscle mitochondria (sarcosomes) and are concerned for the most part with the general problem of mitochondrial function have been reviewed by Slater (5). Harman (27), who reserves the term "sarcosome" for a smaller non-mitochondrial fraction of muscle, finds that components which have both acceleratory and inhibitory action on mitochondrial oxidative processes can be isolated from this small granule fraction. Similar stimulatory properties have been reported for the supernatant solution from housefly flight muscle homogenates (28). Both these findings may be of significance in explaining the failure to obtain oxidative phosphorylation with mitochondria from pregnant rat uterine muscle (29, 30). ATPase activities of aqueous extracts of white and red muscles reflect the relative abundance of granules in these two types of muscle (31).

Differences in the concentration of sodium and potassium in heart mitochondrial membranes (32) may be related to the property possessed by the intact mitochondria of actively transporting potassium but not sodium (33, 34).

CONTRACTILE SYSTEM

Localization of components in the contractile system.—The quantitative data available support the view that myosin is localised in the A band (35 to 38). Extraction of the A substance brings into solution 95 to 100 per cent of the myosin ATPase (38), the extra protein (35, 38), and some actin; the amounts of actin extracted are reduced in glycerated preparations (38). With myofibrils from *Limulus polyphemus*, the failure to extract myosin selectively has prompted the suggestion that the large filaments in this muscle consist of actomyosin (39). Selective extraction at low ionic strength removes the I band and yields a solution that is free of myosin but contains tropomyosin and a form of inactive actin (37, 38). Careful study of the localization of radioactively labelled adenine nucleotide has indicated the difficulties in relating the findings with frog striated muscle, fixed under various conditions, to the distribution *in vivo* (41, 42). Under conditions in which fixation artifacts are presumed to be minimal, concentration of the nucleotide was shown to occur in the A band near to the boundary with the I band (42a). A relatively small proportion of the adenine nucleotide, which probably corresponds to the bound nucleotide of the myofibril, is responsible for the ultraviolet dichroism of frog muscle (42).

Interesting studies with the fluorescent antibodies suggest that the L- and H-meromyosins are localized, respectively, at the lateral edges and in the central regions of the A band (43, 43a). Such a distribution is difficult to relate to present ideas of structure of the myosin molecule and of the myofibril and to the results of earlier studies with myosin antibodies. Interpretation of these striking findings must await further studies on the antigenic properties of myosin.

Evidence is accumulating for the presence of a continuous two-filament system in invertebrate smooth muscle (44 to 47a), but there is less precise information as to where the contractile proteins are located in this tissue. Direct extraction experiments (47, 47a) suggest that, in molluscan tonic muscles, myosin is localized in the fine filaments, which are cross-linked to the thicker tropomyosin-A-rich filaments (47, 48). The exact location of actin, tropomyosin-B, and myosin in these muscles awaits clarification.

The demonstration that the enzyme that hydrolyses thiol acetic acid, and possibly choline esterase, is localised in the region of the M line (49, 50) raises the question of whether this enzyme may be concerned in conducting the contractile impulse to the myofibril.

Actin.—Although apparently 100 per cent polymerisable in the hands of some workers, actin obtained by the ultracentrifugation method (51) often contains tropomyosin (52) and possibly other impurities (53). Because of its unusual physical properties, it is difficult to establish precise criteria of purity for this protein. The figure of one gram molecule of C-terminal phenylalanine per 56,000 gm. of protein obtained by the carboxypeptidase method (52) confirms an earlier chemical study (54), but the significance of the smaller amounts of serine and iso-leucine, which are also liberated by carboxypeptidase, is not known. Results for the molecular weight of actin vary according to the history of the protein and the method used; e.g., values in the range 50,000 to 80,000 are obtained by osmotic pressure and 100,000 from light-scattering studies on G-actin (55, 56). The latter method gives 3 to 8×10^6 for the apparent molecular weight of the kinetic units of F-actin (57, 58). Many of the properties of F-actin in solution are well-explained by assuming that there is present a network structure of semiflexible F-actin filaments, each composed of many G-actin molecules (57). At low shear rates the network linkages are broken and the F-actin filaments are completely oriented.

The events occurring during the stages of F-actin formation from G-actin (55, 56, 57, 59) can be explained by the theory of fibrous condensation of globular and fibrous aggregations of charged macromolecules (60). It is considered that divalent cations do not take part in the binding between actin molecules but effect the G to F transformation by decreasing the charge on the actin molecules through adsorption (55). In accelerating the polymerization of G-actin, H-meromyosin resembles myosin, but analysis of the effect suggests that the action is not catalytic (61).

The extent of conversion of G-actin into F-actin depends on the magnesium concentration (55, 56, 62). G-actin will not polymerize below a critical concentration which depends on the amount of magnesium present and falls to a low value at magnesium concentrations of 2 to 3 mM (55, 56). On the other hand, Martonosi *et al.* (62) find, contrary to the definition of the critical actin concentration as presented by the Japanese group, that the ratio of G- to F-actin is constant for a given magnesium concentration over a wide range of actin concentration. These discrepancies may arise from the prob-

lems involved in using the ultracentrifugation method to assay the amount of F-actin in a system containing equilibrium amounts of the F and G forms.

The presence of discrete amounts of ADP and ATP in close association with F- and G-actin, respectively, has in the past stimulated workers to suggest a special role for these nucleotides in myofibrillar function. Nevertheless, it has been reported (63) that brief extraction of muscle acetone fibre gives actin preparations that are free of ATP and reversibly polymerisable by simply changing the ionic conditions. If this observation is confirmed, some reorientation of ideas on the significance of the bound nucleotide will be necessary.

So far it has not yet been possible to demonstrate with isolated myofibril preparations the transfer of ^{32}P to the bound nucleotide (64). Likewise, investigation *in vivo* with ^{32}P -labelled inorganic phosphate has shown that for some hours at least the actomyosin-bound ADP of various animals is much less active than the ADP pool of the cell (65). The slow equilibration process is not speeded up by exercising the muscles; this would imply that the G to F transformation is not related to the contractile process. These results are consistent with the view that actin is present in the myofibril in the F form as are the investigations of Barany *et al.* (66, 67) on actin obtained without prior extraction of myosin.

The bound nucleotide of G-actin can be dephosphorylated by H-meromyosin and then phosphorylated again by the creatine phosphokinase system. Once polymerisation of the actin occurs, however, the nucleotide no longer becomes available to these systems (68, 69). The bound ATP of G-actin also readily exchanges with radioactive ATP (65, 70), but neither ADP nor ATP will exchange with the protein-bound nucleotide associated with F-actin (65). Despite this observation and Strohmman's (68, 69) finding that the bound nucleotide of F-actin is not available to the creatine phosphokinase system, prolonged treatment of F-actin with this phosphorylating system in the absence of added nucleotide depolymerises it to the G form with the simultaneous conversion of the bound ADP to ATP. In addition to the inorganic phosphate produced during the polymerisation of G-actin, a slow liberation of phosphate occurs in partially polymerised actin systems after equilibrium has been reached (55, 71). This phosphate is derived from the bound nucleotide, and it has been further claimed that added ATP can be dephosphorylated slowly by equilibrium mixtures of G- and F-actin (55). The suggestion that dephosphorylation is a consequence of F to G and G to F transformations constantly taking place in equilibrium systems (71) is not supported by following the exchange of the ^{14}C -labelled ATP in such a system (72). A possible explanation may be that phosphate production is attributable to nucleotidases which can be demonstrated as impurities in actin preparations (73).

The question of the extractability of actin from smooth muscle is not yet resolved. Although the earlier report (74) that salt extracts contain considerable amounts of free actin has received some confirmation (75), two groups

of workers (76, 77) have been unable to detect the presence of such large quantities of actin in these extracts.

Myosin: molecular weight and size.—Corrected molecular weights of approximately 420,000 are obtained for rabbit L-myosin by the Archibald method (78, 79), whereas sedimentation diffusion measurements (78, 80, 81) give higher results which range from 420,000 to 540,000. These values are appreciably lower than the previously accepted figure of 840,000 (82), as are the 530,000 and 590,000 for cod myosin obtained by Connell (83), who used the sedimentation-diffusion and Archibald techniques respectively. The higher values obtained for the sedimentation constant and its previous reported temperature dependence were probably attributable to the presence of irreversibly formed myosin aggregates (81). On careful analysis, the S_{20w} of the myosin monomer is reproducible at ionic strength 0.6 over the range 1–30°C. and from pH 6 to 9. It is also unaffected by solvent composition, ATP concentration, and one to three reprecipitations of the myosin (80, 81). The sedimentation data available for myosin isolated from *Pecten maximus* smooth muscle seem compatible with the data for vertebrate myosins (84, 85), but dog heart myosin is reported to possess an appreciably lower molecular weight of 250,000 (86).

Both rabbit (87) and certain fish myosins (88 to 91) undergo aggregation which occurs by a stepwise process of molecules joining together side to side to form aggregates up to at least eight times the monomer weight. This aggregation occurs without any appreciable intramolecular change (88, 89), and disulphide bonds are not involved (87, 88, 89).

Independent investigations (80, 92, 93) suggest that the myosin monomer is rodlike in form approximately 1600×25 Å. On the basis of hydrodynamic studies, Kielley & Harrington (94) conclude that the molecule consists of three tightly coiled β -helix polypeptide chains twisted into a stranded structure.

Myosin: subunits.—Standard preparations of rabbit L-myosin contain recognizable contaminants, such as 5'-adenylic deaminase (95, 96, 97), choline esterase (98, 99, 100), and a ribonucleoprotein fraction, which can, at least in the case of the last, be completely removed by chromatography on diethylaminoethyl cellulose (101). For this reason, it is not easy to exclude the possibility that fractions isolated from myosin in less than 15 to 20 per cent yield may arise from contaminating proteins rather than the myosin molecule itself. Such reservations would apply to the protein obtained by treatment with 0.1 M sodium carbonate (102) and probably to some of the fractions isolated after heat treatment (103). The observation (104) that three components of different sedimentation velocities can be recognized in myosin solutions treated with 8 M urea is compatible with the views of Kielley & Harrington (94); the proportions of two of the components are relatively small, however, and some reservation as to their significance may also apply here.

Earlier concepts of the number of L-meromyosin and H-meromyosin

subunits derived from a single myosin molecule need re-examination in the light of studies (105, 106) which give values of 126,000 and 324,000 for the molecular weights of the L and H forms respectively. If these values are taken together with preliminary light-scattering results for the molecular lengths (106, 107), it follows that the myosin molecule consists of one molecule each of L-meromyosin and H-meromyosin placed end to end. The remarkable finding by Middlebrook (108, 108a) that meromyosins with identical C- and N-terminal groups are produced by proteolytic enzymes of different specificities has prompted the suggestion that the process involved is nonhydrolytic. On the other hand, as might be expected from the known specificity of trypsin, C-terminal lysine has been detected in L-meromyosin prepared by short digestion of myosin (109). During the tryptic digestion of myosin, two classes of peptide bonds are split simultaneously at different rates (110). The myosin molecule is thought to consist of a small number of polypeptide chains arranged parallel to one another. In certain places where the chains are arranged in a somewhat random fashion, the peptide links are highly susceptible to proteolytic enzyme attack, whereas in the more ordered regions the peptide links are attacked slowly (110). Much of the original helical configuration of the myosin backbone is preserved in meromyosin formation because little change occurs in the optical rotation during the early stages of tryptic digestion (110, 111).

Spectrophotometric studies indicate that hydrogen bonding between tyrosine side chains and some acceptor group may be much more important for stabilising the tertiary structure of L-meromyosin than of H-meromyosin (112). Evidence of the heterogeneity of H-meromyosin preparations has continued to accumulate both from ultracentrifuge (105, 113) and from chromatographic analysis (114). Unless care is taken to ensure that exactly the same number of peptide bonds are broken, it is unlikely that all the H-meromyosin preparations studied are completely identical. Chromatography on diethylaminoethyl cellulose (114) effectively removes the slower sedimenting components which are often present in H-meromyosin preparations. Further digestion of chromatographically purified H-meromyosin gives rise to several components of lower sedimentation constant than the original H-meromyosin (114a). Under these conditions relatively little loss in ATPase activity occurs. A cysteine-containing peptide, which may be related to the active centre, has been isolated after more prolonged tryptic digestion of myosin (114b). L-meromyosin preparations have also been further fractionated by chromatography (114) and ethanol treatment (115).

The H-meromyosin antigen can be recognized in myosin preparations (116). This and other immunochemical studies (43, 117) show considerable promise for the investigation of the fundamental structure of the myosin molecule.

Actomyosin.—The orthodox view that ATP dissociates actomyosin or myosin-B into actin and myosin has been supported by the isolation of actin from myosin-B (118) and the demonstration that ATP is essential for actin

to be preferentially centrifuged out of solutions of actomyosin (119, 120). Reinvestigation of the interaction by the Morales group (121, 122, 123) has indicated that 5 hr. myosin contains about 65 per cent of L-myosin monomer and 35 per cent of two types of larger particles of molecular weight 5×10^6 and 10 to 50×10^6 . In other cases, some extracts may contain as much as 90 per cent or more of myosin-A (124). Although the most recent investigations (123) confirm earlier light-scattering observations, from which it was deduced that the weight average molecular weight is unchanged, the Morales group now concedes that ATP and inorganic pyrophosphate bring about dissociation of the particles intermediate in size and that "some cementing substance (possibly actin)" may be involved (122). Holtzer *et al.* (125) clearly find that actin is a product of the action of ATP on myosin-B.

There is no information on the nature of the heaviest particles found in 5 hr. myosin which inflate at constant molecular weight in the presence of ATP (122, 123). The results obtained by the use of light scattering by the Morales group are still at variance with those of Gergely (126), who concluded that the average molecular weight of myosin-B particles was reduced three- to sevenfold on addition of ATP. Differences in the mode of preparation of myosin-B or changes that occur on dilution may explain this discrepancy (122). Gergely considers that the results of light-scattering studies on the interaction can be interpreted to indicate that actomyosin is formed and dissociated in a stepwise fashion (127). Although some of the assumptions made in this particular analysis may not be acceptable to every worker in the field, the general conclusions from all the investigations reported here confirm that ATP brings about the dissociation of actomyosin into actin and myosin.

It has been confirmed also that actin is an essential component for the concentration of actomyosin threads (128, 129), and an earlier claim that actin-free myosin threads could respond to ATP in a specific manner has been withdrawn (130). The report that certain dyes and nucleic acid can replace actin in this system (131) merits consideration, for such systems might provide useful models for studying the function of actin.

Inorganic pyrophosphate competes with ATP in the dissociation of actomyosin solutions (132), but little binding of the inorganic ion occurs when it induces physical change in the actomyosin system (133). This finding does not support the hypothesis that changes in the myosin B-system are brought about by the increase in electrostatic repulsion consequent on the adsorption of polyphosphate anions. Ca will not replace the Mg required for the dissociation of actomyosin by pyrophosphate at 25° (132).

Increased interest in the comparative aspects of muscle biochemistry has provided impressive evidence of the universality of the actomyosin system (12, 134, 135, 138). Considerably less actomyosin can be extracted from cardiac and stomach muscle (136, 137) and from uterus (136, 137, 138) than can normally be obtained from skeletal muscle. Differences in solubility between species are also often apparent (24); for example, actomyosin is

extracted from codling at ionic strengths as low as 0.1 (90), and actomyosin solutions can be obtained from mantle muscle of the squid by repeated extraction with distilled water (139). In the latter case, the complex has been called M-actomyosin to indicate certain differences in properties which distinguish it from the corresponding rabbit protein. It is possible that some of these differences may be attributable to the relatively large amounts of tropomyosin that are present in the original extract and are not readily removed by purification procedures (139). Mueller's finding (140) of an increased solubility of actomyosin at low ionic strength in the presence of ATP and relaxing-factor preparations may be of significance in explaining some of these species differences.

Extraction of muscle from cold-blooded vertebrates and invertebrates under the Weber-Edsall conditions gives actomyosin rather than myosin (90, 139, 141). The role of arginine and a protein factor, reported earlier by the Tonomura group (142) to be essential factors for the viscosity recovery after ATP addition to actomyosin-isolated pecten adductor muscle, has not yet been clarified. Arginine breaks up the labile structure set up in pecten actomyosin in the early stages of recovery after ATP addition; however, the reaction does not appear to be particularly specific to this substance.

Ion binding.—Redetermination of the Mg content of myosin-B gives an average value of 0.56 $\mu\text{g}/\text{mg}$ (143). This value is somewhat lower than has been reported earlier, but none of this magnesium is removed by washing the myosin with ethylenediaminetetraacetate. Myosin and its subunits obtained by tryptic digestion are amongst the proteins with the strongest affinity for Ca and Mg (144). Above pH 4.5 the affinity of the L- and H-meromyosins for both ions is similar. In whole muscle the bulk of the calcium is bound to protein (145), whereas most of the magnesium is free (145). In view of the inhibitory action of Ca on the relaxing factor, it might be expected that all of this ion is bound in the relaxed state.

Myosin ATPase.—When L-myosin is chromatographed on diethylaminoethyl cellulose, at least two peaks of ATPase activity can be recognized (101). This enzymic heterogeneity appears to be a feature of the myosin ATPase, for when H-meromyosin is studied in a similar fashion (114) the specific ATPase distribution along the eluted peak is more consistent with that of a homogenous enzyme preparation. Under certain conditions, part of the myosin is not held by the diethylaminoethyl cellulose, and further amounts can be eluted on increasing the ionic strength (101, 146). Brahm has called these two fractions α and β and finds the ATPase activity higher in the latter. To avoid confusion with Dubuisson's α - and β -myosin terminology for the electrophoretic components of crude preparations of the protein, in this review the chromatographically separated components will be referred to as myosin fraction α and myosin fraction β . In the studies reported by Perry, the more readily eluted fractions were often lower in ATPase activity, but there was evidence that this was at least in part attributable to the presence of contaminating proteins in myosin preparations. Myosin fractions α and β

are interconvertible (147). Although it was considered earlier (146) that fraction β had a higher molecular weight, the most recent view is that the molecular weights of the fractions are identical and that fraction β is a more extended form of myosin with higher ATPase activity (147). Enzymic heterogeneity may be a widespread property of myosin preparations, for similar findings have been reported with chick embryonic myosin (148). Rat uterus actomyosin has a relatively low ATPase activity which is increased to a level comparable with that of the skeletal protein after treatment with trypsin (77). Chromatography may yield important new facts about the myosin molecule, but certain special features about its behaviour under these conditions need further investigation.

The results of chromatography add to the complexity of the myosin ATPase system, a situation reflected annually by the number of papers dealing with some aspects of this field. For a given ATP level, the addition of the creatine phosphokinase system reduces the activities of the Mg-activated adenosine triphosphatases of myosin-A and -B (149). Replacement of the oxygen atom between the two terminal P atoms of ATP by a methylene group, the phosphonic acid analogue, renders the nucleotide unhydrolysable and without effect on actomyosin gels (150). Likewise, adenosine tetraphosphate is unable to induce contraction in actomyosin systems, although it is hydrolysed slowly by actomyosin (151). Increasing the polyphosphate chain length further decreases the rate of hydrolysis. Certain organic solvents have now been added to the list of reagents that stimulate myosin ATPase (152).

With the possible exception of uridine triphosphate, myosin ATPase hydrolyses all the nucleotide triphosphates at high rates, but pronounced differences occur in the presence of certain organic sulphydryl reagents as well as ethylenediaminetetraacetate, Mg, and DNP (153, 154) according to whether or not the nitrogen base of the nucleotide is substituted in the 6 position with an amino group. The results obtained in the case of ATP and cytidine triphosphate with sulphydryl reagents (153, 155) extend earlier studies by other workers and can be explained by the assumption that two thiol groups are involved at the active centre. One of these groups is concerned in the hydrolysis of all nucleotide triphosphates, and combination with it inhibits this reaction. The other group interacts in some way with the 6-amino group of purine and pyrimidine nucleotide triphosphates and thereby slows down the rate of hydrolysis. Combination of this thiol group, which occurs in the presence of low levels of *p*-chloromercuribenzoate, phenylmercuric acetate, cysteine ethyl ester, or Cu (153), stabilises a favourable configuration, and the hydrolysis rate is increased. Consistent with this view is the conclusion drawn from studies on the stimulating action of DNP on myosin ATPase that this phenol acts by decreasing the binding of the amino group on the purine or pyrimidine ring to the enzymic site (156). Further evidence of some affinity of DNP for the groups on the myosin molecule involved in binding the 6-amino group of nucleotide triphosphates comes from analysis of Arrhenius plots of magnesium-catalysed hydrolysis of ITP

and ATP (157). Substrates lacking an amino group form an enzyme substrate complex which is very sensitive to change at 16°C. With ATP as substrate, this effect is not observed, but in the presence of DNP the Arrhenius plot resembles that for ITP.

Treatment of myosin with high concentrations of iodoacetamide destroys in parallel both enzymic and actomyosin-forming properties, whereas if actomyosin is acted upon in a similar way only the enzymic property is lost (158, 159). If it can be assumed that iodoacetamide reacts only with —SH under these conditions, it follows that the myosin molecule possesses two types of —SH groups, one type concerned in the ATPase activity and the other type in the combination with actin. The high affinity of ATP for actomyosin is reduced to the level of that for pyrophosphate by the iodoacetamide treatment; this condition suggests that the pyrophosphate- and actin-binding centres of the myosin molecule are identical (158, 159).

The characteristic rise in ATPase activity above pH 7.0 of myosin (160) is abolished by treatment of myosin with H_2O_2 , a procedure that has little effect on the pH optimum of the enzyme at 6.5. These results imply involvement of —SH groups in the rising phase of activity in alkaline range. Comparable studies by Gilmour (161) with *p*-chloromercuribenzoate and DNP employed at levels to stimulate the myosin ATPase indicate that these substances have similar effects, different from those of H_2O_2 , on the pH activity curve. The optimum at pH 6.5 is eliminated, and stimulation by *p*-chloromercuribenzoate and DNP occurs only at higher pH values.

Cysteine ethyl ester or *S*- β -aminoethylisothiuronium increases the activation of myosin-B ATPase obtained with Ca (162). In the case of the latter compound (163, 164), the effect is accompanied by a reduction of the *p*-chloromercuribenzoate-titrable groups. Some simplification in the system is achieved by replacing KCl with tetraethylammonium chloride. Under these conditions there is little ATPase activity in the absence of Ca (165).

Under certain conditions high pressures reversibly stimulate the Ca-activated myosin ATPase (165, 166), whereas in other cases the ATPase and actin-combining property are irreversibly inactivated in a parallel way but the myosin remains soluble (167, 168, 169).

On heat denaturation of both myosin and myosin-B ATPase, fast and slow reactions can be recognized (170), a finding which may be related to the observation that acid treatment produces at least two forms of enzymically active myosin with different thermal sensitivities (171).

The initial rate of ATP hydrolysis by myosin or actomyosin may be 30 times as high as the stationary rate reached after 30 to 60 sec. (172). Although Ca is ineffective, Mg or Mn are essential additions to restore this effect to dialysed myosin-B. In clear distinction to the effect of Ca and Mg on the stationary rate of ATPase activity in high salt concentrations, neither of these cations has any effect on the initial phase of undialysed myosin-B (172, 173). Ethylenediaminetetraacetate abolishes the high initial rate (174), but, on the other hand, the effect is still evident, although progressively reduced by increasing concentrations of *p*-chloromercuribenzoate over the range of

concentrations within which this reagent stimulates the ATPase activity.

Mechanism of action of myosin ATPase.—Kinetic analysis (175) of the Ca-activated myosin ATPase system does not permit a differentiation between the mechanism in which free ATP is the substrate and activation occurs by binding metal to the protein (176), and the reverse situation in which the metal ATP compound is the substrate (177). The former hypothesis is preferred since all the data can be explained without further assumptions (176). A study of the non-enzymic transfer of orthophosphate from ATP to a variety of acceptors has shown that small amounts of the alkaline earth metals can have a marked synergistic effect on the rate of such transphosphorylations (178). These findings may be of great significance in understanding some of the properties of the actomyosin system. Earlier results (177) which indicated that traces of Ca or some similar cation might be required for the Mg-activated ATPase of myofibrils have been extended by Weber (179) whose results support the view that the metal is Ca. These special features of the actomyosin ATPase have led Lowenstein to suggest that the active substrate which forms the enzyme-substrate complex for this enzyme is a calcium-ATP-magnesium chelate. This mechanism would explain the requirement for traces of Ca for enzyme activity in the presence of excess magnesium.

Many of the known facts about the myosin and actomyosin ATPases could be explained if it were assumed that acceptable substrates are either Ca-ATP-Ca or Ca-ATP-Mg (178) but that Mg-ATP-Mg is not split by either myosin or actomyosin.

Evidence for the binding of Mg comes from a study of the effects of monovalent salts on the magnesium-activated ATPase of myosin-B (180). This work also provides evidence that the inhibition obtained with Mg at higher ionic strength is not attributable to dissociation of the actomyosin. It may be caused by binding at the centres normally occupied by the enzyme substrate complex.

Although some of the effects of ethylenediaminetetraacetate, e.g., on the Mg-activated actomyosin ATPase at low ionic strengths, may be explained on the basis of the mechanisms described, kinetic studies (181, 182) have not yet clarified the mechanism of the stimulation of ATPase activity obtained with this substance at high ionic strengths and in the absence of added Ca or Mg. It is of interest that inorganic pyrophosphate can activate myosin ATPase under conditions similar to those in which ethylenediaminetetraacetate is effective (183).

Evidence is accumulating for the existence of an intermediate during ATP hydrolysis although little is known of its nature. Ulbrecht and co-workers (184, 185) have demonstrated that exchange of inorganic phosphate takes place between AD³²P and ATP during hydrolysis of the latter by actomyosin systems but not when purified L-myosin is used. This reaction is also catalysed by the granular ATPase per se, but, although the actomyosin preparations were contaminated with this enzyme, the amounts present were considered inadequate to produce the effect. If confirmed, these results imply

a specific role for actin in the formation of a phosphorylated intermediate during hydrolysis of ATP. Although Ulbrecht and co-workers were unable to demonstrate phosphate exchange during hydrolysis of ATP by myosin, Polish workers (186, 187, 188) cite an increase in the inorganic phosphate bound to myosin, meromyosins, and actomyosin during ATP hydrolysis as evidence for the existence of a phosphorylated protein intermediate. A similar conclusion has been postulated to explain the effect of ATP on surface films of natural actomyosin (189). More convincing evidence of the existence of an intermediate both with myosin and actomyosin is provided by Koshland and collaborators (190, 191) from studies of the enzymic hydrolysis in the presence of $H_2^{18}O$. This work indicates that a specific myosin-water interaction occurs, and from the high level of $^{18}O_2$ in the phosphate produced it follows that an exchange between an intermediate and the $H_2^{18}O$ had occurred at some stage of the hydrolysis. It is significant that this exchange can be demonstrated with Mg but not with Ca as activator.

Tropomyosins-A and -B.—An increased interest in the biochemistry of invertebrate smooth muscle has resulted in the recognition of the existence of two types of this protein, tropomyosin-A (insoluble at low ionic strength) and tropomyosin-B (soluble at low ionic strength). These proteins are classified together because of certain common features of amino acid composition and physical properties (192 to 195). Tropomyosin-B was first isolated by Bailey from rabbit muscle, where it represents at least 10 to 12 per cent of the total myofibrillar protein (196), and is probably present in all types of muscle (194, 195, 197, 198).

Bailey (192, 193, 199) and the Laki group (194, 195, 200, 201) have isolated tropomyosin-A from the muscles of molluscs, annelids, and cephalopods. This protein, widely distributed in invertebrate muscle, represents the main component of the tonic portion of molluscan adductor muscle (199, 202) from which in some cases tropomyosin-B has also been isolated (192, 194, 195, 199).

Redetermination of the molecular weight of rabbit tropomyosin-B by light-scattering measurements gives a value 20 per cent lower than the earlier osmotic pressure measurements, i.e., 52,900 at $\mu=1.1$ rising to 100,000 at $\mu=0.1$ (203). Aggregation as ionic strength decreases proceeds by an end to end process to at least a hexamer stage. The sedimentation behaviour of tropomyosin-B from vertebrate and invertebrate sources is remarkably similar (194), and from amino acid analysis can be calculated a minimum molecular weight of 62,000 which is characteristic of this protein irrespective of its source (195). Although the general pattern of amino acid composition of tropomyosin-B is constant, small variations in individual amino acids occur which are characteristic of the species from which the protein is derived (194, 204, 205). The C-terminal amino acids are not identical in all tropomyosins-B, but the rabbit (54, 195) and probably the frog protein (204) have C-terminal serine and isoleucine. A constant feature of native tropomyosin-B is the occurrence of two —SH groups per molecule (195). On the basis of the

proline content, it is concluded that the minimum molecular weight of tropomyosin-A is 60,000 (201).

Sedimentation diffusion and light-scattering studies give values of 131,000 and 137,000, respectively, for the molecular weight of *Pinna nobilis* tropomyosin-A (206). The difference between chemical (201) and physical measurements cannot be explained by assuming that dimerisation occurs, since the molecular weights in 8 *M* urea and water are identical (207).

The suggestion, originally made on the basis of several lines of evidence (192, 193, 202, 206), that tropomyosin-A is responsible for the characteristic electron-microscope and x-ray diffraction patterns of paramyosin fibrils has been confirmed by the demonstration that paramyosin fibrils and isolated crystals of tropomyosin-A take up electron strain in an identical way (208). Paramyosin fibrils possess ATPase activity of the myosin type, although of much lower intensity, but the products of tryptic digestion are different from those obtained with myosin (209).

Immunochemical studies suggest similarities between *Venus mercenaria* tropomyosin-A and the L-meromyosin moiety of vertebrate myosin (210). Laki (210) uses this observation to support his view that, although tropomyosin-A cannot be isolated from vertebrate muscle, it is present in this tissue as a subunit of myosin. By digestion of a suspension of previously denatured myosin with trypsin, a soluble subunit has been isolated and crystallised (211). On the evidence provided, this protein is very similar in properties and composition to the conventional L-meromyosin preparations, but nevertheless, for reasons which are not too convincing, Laki classes it as a tropomyosin-A.

The large amounts of tropomyosin-A in molluscan adductors have led to much speculation on the role of this protein in relation to the adductors' tonic properties (199, 212). ATP has a plasticising action on the tropomyosin-A fibrils of the smooth adductor of *Pecten maximus*; this interaction is counteracted by Ca and Mg (213, 214, 215) and, according to Ruegg (215), may be of significance in explaining the tonicity of these muscles. Another view (216, 216a, 216b), however, is that the tension could be maintained by the crystallisation of tropomyosin-A which can be shown to occur at pH 6.5. Whatever the explanation, in those sections of the tonic portion of *Pecten adductor* where resistance to stretch is overwhelmingly greater than active tension, tropomyosin-A is especially abundant (215).

Minor protein components of the myofibril.—Probably at least 5 to 10 per cent of the myofibril consists of proteins other than myosin, actin, and tropomyosin (12). Some of the protein preparations recently isolated and claimed to be new myofibrillar components are far from homogenous, and their relation to this as yet undefined fraction is frequently not clear. The pseudoglobulin extracted from myofibrils at pH 8 to 9 and low ionic strengths has been identified as a form of inactive actin (37). Tsao and co-workers (217, 218, 219) have isolated a globular, water-soluble protein, the amino acid composition of which is very similar to both actin and creatine phospho-

kinase. It is difficult to decide if protein obtained in low yield from the myofibril is a true myofibrillar component rather than a contaminant of different origin. For example, appreciable amounts of protein, component C (37), resembling the sarcoplasmic proteins in electrophoretic and chromatographic behaviour (37, 220) can be extracted from repeatedly washed myofibrils. By ion-exchange chromatography, the extra protein fraction, sometimes called the X protein (35), has been resolved into four main fractions, each possibly complex in themselves (220). The extra protein probably corresponds to the (221, 222) fraction T of Ivanov and co-workers which is a relatively constant fraction in skeletal, cardiac, and smooth muscle. The globulin (220) and water-soluble proteins which have been isolated from the extra protein may be related to the fractions reported by Tsao and co-workers (217, 219). Most of the ribonucleoprotein of rabbit skeletal muscle (223) and rat myometrium (224) sediments with the myofibrillar fraction. A similar nucleoprotein can be isolated from the extra protein (220, 223) and from myosin (101) and very likely is the form in which the nucleic acid is associated with tropomyosin in nucleotropomyosin. The relation of metamysin to myosin is not yet clear, but it does show some similarities to γ -myosin. This latter fraction is heterogenous and resembles actin in its salting-out range with ammonium sulphate and in its interaction with myosin (225, 226).

Metamysin contains at least two major protein components (227 to 231) and nucleic acid. The Δ -protein isolated by the Amberson group (232, 233, 234) is likewise complex. Tropomyosin is usually present in this preparation (201, 233), and one component complexes with myosin and actomyosin, at the same time dissociating the latter complex.

CONTRACTION AND ATP SPLITTING

The measurement of ADP levels *in situ* by a highly sensitive spectrophotometric technique indicates that only 2 to 3 per cent of the expected increase in this nucleotide occurs during contraction of perfused frog sartorius muscle (235). The importance of this experiment is not that, as the authors conclude, the amount of change is too small for ATP to be a source of energy during contraction but that the effect is additive and that sufficient change occurs in the sarcoplasmic ADP to make its effect felt through the semipermeable membrane of the mitochondria within the time course of a single twitch. This implies that the actual changes in ADP concentration close to the myofibril will be very much greater than those measured by the spectrophotometric method, for appreciable rephosphorylation by sarcoplasmic phosphokinases surely must occur.

Although no significant change in nucleotide and creatine phosphate levels could be demonstrated during a single twitch of turtle retractor penis muscle, $0.5 \pm 0.11 \mu\text{M}$ inorganic phosphate was liberated per gm. of muscle (236). The free energy of hydrolysis of ATP in the conditions applying in the living cell is probably 11 to 12 kcal. (237), from which it follows that the inorganic phosphate liberated in a single twitch in the experiments of Davies

et al. (236) would be about adequate to account for the work done. Carnosine phosphates have been excluded as possible phosphate donors in contraction (238).

It is possible that the apparent constancy of the organic phosphate levels during a single twitch is attributable to the failure to exclude phosphorylation by anaerobic glycolysis. Such a viewpoint is supported by the finding that creatine phosphate breakdown in amounts related to the work done accompanies short tetanic contraction of iodoacetate poisoned frog sartorius muscle (239). Creatine phosphokinase is not inhibited by concentrations of iodoacetate adequate to stop glycolysis (240).

Studies on ^{32}P distribution between the inorganic phosphates of muscle do not give any support for the view that muscle activity is accompanied by a rapid hydrolysis and reformation of ATP. Activity does not produce redistribution of phosphate between ATP and creatine phosphate (241); furthermore, there is no significant speed-up in the approach to an equilibrium distribution of radioactivity between the α , β , and γ P atoms of ATP in repetitively contracted muscle as compared to a resting control (242, 243). The specific activities of the organic phosphates reported in these investigations are very low compared with that of the extracellular inorganic phosphate. For this reason and the probability that the various organic phosphates in the cell are localized at different points in the cell and are not necessarily in metabolic equilibrium, it is difficult to assess the real significance of these results (cf. 65).

The ATPase activity and mechanical properties of glycerated fibres from psoas (244, 245) and from heart (246) do not always respond in a parallel manner to changes in the ionic environment. Energetic considerations, however, lead to the conclusion that the free energy liberated by ATP hydrolysis during contraction is at least equal to the work done (247). Several other studies on phosphate turnover and contraction in the glycerated fibre system have been reported (248, 249).

RELAXING FACTOR

High-speed centrifugation of muscle homogenates from which myofibrils have previously been removed sediments granular material which inhibits the myofibrillar ATPase and decreases the tension in model systems contracted by ATP (250, 251, 252). Such relaxing factor preparations cannot be replaced by phosphokinase systems (253, 254, 256, 257, 258), the relaxing effects (259) of which are undoubtedly attributable to their action in facilitating the penetration of ATP into the centre of glycerated fibres which already contain relaxing factor (253). There is some evidence, however, that when ATP diffusion is not limiting the activity of minimal amounts of the relaxing factor preparations can be potentiated by transphosphorylating systems (260). It seems clear that the effect of relaxing factor on tension in model systems and on the ATPase are complementary aspects of its function, for the results in one system have so far been paralleled by those in the other. Given that contraction and relaxation are the consequences of high and low

rates of ATP hydrolysis, respectively, it follows that the primary action of the relaxing factor may be to inhibit the Mg-activated myofibrillar ATPase. Intact myofibrillar structure is not itself a prerequisite, since its action as the factor inhibits both the superprecipitation of natural actomyosin (140) and enzymic activity of natural and of synthetic actomyosin (140, 261). In the presence of relaxing factor, superprecipitated actomyosin can be rehydrated by ATP concentrations similar to those present in the cell (140).

The cytological entity with which the factor is associated *in situ* is far from clear. If preparations are tested in the absence of oxalate, factor activity has usually only been reported to be present in the smaller granular fraction which sediments after prolonged centrifugation at high speed (255, 258, 260). For this and apparently no other reason, it has been assumed that the factor is microsomal in origin (262). Relaxing activity, however, is present in other fractions, for the heaviest granule fraction of rabbit muscle, which is rich in mitochondria, has the highest specific activity when assayed in the presence of oxalate (261). In pigeon breast muscle the most active granules are those of intermediate size and possessing relatively low oxidative activity (261). Unlike the microsome fraction of other tissues, the material isolated by high-speed centrifugation of muscle suspensions is low in ribonucleic acid (223) and probably corresponds to the sarcoplasmic reticulum (263) with which far fewer ribonucleoprotein granules are associated than with the endoplasmic reticulum of other cells. This reticulated structure in muscle may play a part in conducting activation from the membrane into the interior of the cell (264) and may not be strictly comparable to the endoplasmic reticulum.

When assayed in the presence of oxalate, relaxing-factor activity can be qualitatively recovered by sedimentation from muscle extracts (252). The Gergely group (265, 266) reports that granules sedimented from extracts require the addition of supernatant to restore full relaxing activity in tension and enzymic experiments. The cofactor suggested by these studies is dialysable and can be removed from dialysates by charcoal or Dowex-1. It can be replaced by pyrophosphate (267), and, in view of the similar potentiating action of pyrophosphate and oxalate in the relaxing-factor system (261), the latter substance might also be expected to substitute for the dialysable factor. Nevertheless, with some granule preparations assayed at low concentration in the presence of oxalate, cofactor is still required (265). The increase in cofactor requirement on incubation (256) resembles the loss in relaxing-factor activity of granule preparations obtained on ageing (261). This decrease in activity is completely restored by oxalate and suggests slow liberation of some substance that is inhibitory to the relaxing factor (possibly Ca) and is in an available form during ageing of the preparations.

So far attempts to solubilise the relaxing factor by treatment with acetone or deoxycholate (256, 258, 261) have led to inactivation; nor can soluble preparations be obtained by salt extraction (261). Likewise, the action of phospholipase C on *Clostridium welchii* (251) destroys factor and ATPase activity simultaneously. Although pyridoxal phosphate resembles the relax-

ing factor in that it inhibits the myofibrillar ATPase and this inhibition is relieved by carnosine, it does not seem to be a component of the relaxing system (268).

The striking feature of relaxing factor action is that in some way an apparently insoluble granular system exerts effect on the myofibril which is likewise insoluble. The mechanism produces problems, but the important discovery that a granule-free relaxing substance (269) which is soluble and dialysable (270) can be produced on incubation of granules with ATP may lead to a better understanding of the mechanism. Marsh (271) has identified the relaxing factor as being composed of α -glycerolphosphate and orthophosphate, but it has yet to be established whether this system is identical to that of Briggs & Fuchs (270). Suggestions that myofibrils incubated with ATP and granules result in the activation of the latter (272) also hint at the removal or extraction of some substance from granules.

The marked effects of low concentrations of Ca in the system and the requirement of low traces of a similar cation by the Mg-activated myofibrillar ATPase of myosin (177) have prompted the suggestion (8, 177, 179) that relaxing-factor action may be to inhibit the ATPase by binding this Ca or similar cation. In a striking way, the inosine triphosphatase activity and relaxing action are much less affected by both ethylenediaminetetraacetate and the relaxing factor (177, 261, 273). This suggests that the amino group at position 6 of the purine ring may be involved in the requirement for the traces of cation essential for the Mg-activated ATPase.

Oxalate itself has a high affinity for Ca, and it is difficult to explain on the basis of the Ca-binding hypothesis why the factor activity should be potentiated by this substance which is relatively ineffective itself and which protects the system from Ca inactivation. Gergely & Parker (269) find that, although the granules combine with Ca, the interaction of their granule-free relaxing factor with the myofibrils does not involve the removal of bound Ca. On the other hand, contrary to an earlier interpretation of the data (274), the ability of ethylenediaminetetraacetate and a number of analogues to chelate with Ca correlates well with their relaxing action on glycerated fibres (275).

Although the factor itself is not involved the relaxation of glycerated fibres induced by ethylenediaminetetraacetate in the presence of iodoacetic acid (276), by acetone (277), and by zinc and other bivalent metals (278 to 280a), these systems possess features that may be of significance for understanding the mechanism of the physiological process.

NON-PROTEIN NITROGENOUS COMPOUNDS

Phosphagens.—A timely review by Ennor & Morrison (281) summarises the present position of biochemistry of phosphagens and related guanidines, which in recent years have been the subject of study in several laboratories (282 to 286). The earlier reported enzymic conversion of creatine phosphate to creatinine in the presence of glucose-1-phosphate (287) has not been confirmed (288). In vitamin-E deficiency there is an increase in creatine incorpo-

ration into skeletal muscle (289) but no net increase in creatine synthesis (290). A decrease in the transfer of phosphate from creatine phosphate to glucose-6-phosphate (291) also occurs in this deficiency. Creatine can be phosphorylated by a soluble enzyme system from rat muscle by using 1:3 diphosphoglycerate in the absence of added ADP (292, 293). The ability of nicotinamide-adenine dinucleotide (NAD)³ to replace ADP as an essential addition in a similar system in which phosphoenolpyruvate is the donor seems to be the consequence of ADP formation from NAD by enzymes present in rat muscle extracts.

The values for the creatine phosphate content of mammalian heart vary according to the techniques employed (294). With improved methods of removal and analysis, one-half to two-thirds of the total creatine has been reported to be present in the phosphorylated form (294, 295). The heat of hydrolysis of creatine phosphate, $\Delta H = -9.0 \pm 0.5$ kcal. per mole, is appreciably higher than that of ATP (296).

Carnosine and anserine.—The effects of carnosine on the metabolism of muscle systems reported by the Severin school have been explained as being caused by the buffering action of this peptide (297) or its ability to chelate metal impurities (298, 299). Although carnosine itself is ineffective, the methyl ester does produce some stimulation of the respiration of frog muscle homogenates (300). Meshkova (301) reports that anserine has a protective action on the oxidative phosphorylating system in muscle which is not attributable to its buffering action and which cannot be replaced by histidine. As there is some correlation between the amounts of carnosine and anserine in muscle and the extent to which the tissue can function anaerobically, Davey (299, 302) has suggested that these peptides have a buffering function *in situ*. Carnosine probably occurs in the free form in muscle homogenates (303).

Studies on the carnosine-anserine synthetase system of chick pectoral muscle (304 to 307) are compatible with the conception of enzyme bound β -alanyladenylate as an intermediate. In vitamin E-induced dystrophy, decreased synthesis of anserine is found in rabbit muscle (308). Gastric mucosa is the only tissue of the rat other than muscle that contains carnosine; carnosinase activity, however, is present in most tissues (309).

Nucleotides and nucleic acids.—Reinvestigation has confirmed the classical view that adenine compounds are the source of the ammonia evolved from muscle after death (310, 311). Deamination occurs rapidly in carp muscle, even at -8°C . (312, 313), but small amounts of ATP and ADP, presumably the bound nucleotide of the myofibril, and ITP and IDP remain after the onset of rigor (310, 314, 315). 5'-Adenylic deaminase is not present in certain invertebrate smooth muscles (316, 317), although adenosine deaminase has been reported in squid muscle (317). Both vertebrate (312, 318, 319, 320) and

³ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN).

invertebrate muscle (317) possess enzymes capable of dephosphorylating nucleotides. Cat heart perfused with adenosine takes up 50 per cent as nucleotide and the remainder appears in the perfusate as inosine and hypoxanthine (320).

The persistent 5'-adenylic deaminase activity of rabbit myosin preparations is undoubtedly attributable to contamination by the enzyme (95, 96, 97, 101) which has recently been crystallised from rabbit muscle (321). Not all myosins show this property since, although pigeon breast muscle (322, 323) is rich in 5'-adenylic deaminase, myosin and myofibrils prepared from it are completely free of the enzyme (322).

The distribution of 5'-adenylic deaminase suggests that it is not an essential enzyme so far as the contractile process is concerned. Nevertheless, changes in the ultraviolet absorption spectrum of living muscle have been interpreted as indicating that the deamination of adenine nucleotide accompanies a single twitch in vertebrate muscle (324). Certainly, on prolonged activity inosine monophosphate can accumulate (314, 315), and vertebrate muscle does contain a system for reaminating the nucleotide to adenosine monophosphate (325 to 328). Studies *in vivo* (325, 326) and with partially purified preparations (325 to 328) indicate that aspartic acid is the source of the amino group and that adenylosuccinate is probably the intermediate in the reaction.

The nucleic acid content of adult skeletal muscle (223, 329) is low, and, apart from some investigation on fish muscle (330, 331), its metabolism has been little studied. Interest in muscle nucleic acid has been centred mainly around that associated with myosin and isolated myofibrils (96, 223, 332, 333) and the changes occurring in uterine muscle during pregnancy (138). Nucleotides are associated also with the myosin nucleic acid fraction (96, 332) which is largely responsible for the phosphorus always present in the usual preparation of this protein. This phosphorus fraction can conveniently be removed by chromatography without impairing the biological properties of myosin (101).

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BIOCHEMISTRY OF STEROID HORMONES^{1,2,3}

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This review will discuss recent work on the biosynthesis of steroid hormones and the pathways and enzymes involved in their transformations *in vivo* and *in vitro*. Emphasis has been placed upon the discussion of new path-

¹ The survey of the literature pertaining to this review was concluded in November, 1960.

² The following abbreviations are used: NAD for nicotinamide-adenine dinucleotide; NADH₂ for nicotinamide-adenine dinucleotide, reduced form; NADP for nicotinamide-adenine dinucleotide phosphate; NADPH₂ for nicotinamide-adenine dinucleotide phosphate, reduced form.

The systematic equivalents of the trivial names used in this review are as follows:

C₁₈: 1,3,5(10)-Estratriene-3,17 β -diol, Estradiol; 1,3,5(10)-Estratriene-3,16 α ,17 α -triol, 17-Epiestriol; 1,3,5(10)-Estratriene-3,16 β ,17 α -triol, Estriol-3,16 β ,17 α ; 1,3,5(10)-Estratriene-3,16 β ,17 β -triol, 16-Epiestriol.

C₁₉: 4-Androstene-3,17-dione, Androstenedione; 3 β -Hydroxy-5-androsten-17-one, Dehydrocandrosterone; 4-Androstene-3,11,17-trione, Adrenosterone.

C₂₁: 5 β -Pregnane-3 α ,20 α -diol, Pregnanediol; 3 β -Hydroxy-5-pregnen-20-one, Pregnenolone; 20 α -Hydroxy-4-pregnen-3-one, 20 α -Dihydroprogesterone; 20 β -Hydroxy-4-pregnen-3-one, 20 β -Dihydroprogesterone; 21-Hydroxy-4-pregnene-3,20-dione, Deoxycorticosterone; 5 β -Pregnane-3 α ,17,20 α -triol, Pregnanetriol; 11 β ,21-Dihydroxy-4-pregnene-3,20-dione, Corticosterone; 17,21-Dihydroxy-4-pregnene-3,20-dione, 11-Deoxycortisol; 21-Hydroxy-4-pregnene-3,11,20-trione, Dehydrocorticosterone; 11 β ,17,21-Trihydroxy-4-pregnene-3,20-dione, Cortisol; 17,20 β ,21-Trihydroxy-4-pregnene-3,11-dione, 20 β -Dihydrocortisone; 11 β ,17,21-Trihydroxy-1,4-pregnadiene-3,11-dione, 20 β -Dihydroprednisone; 11 β ,17,21-Trihydroxy-5 β -pregnane-3,20-dione, Dihydrocortisol (5 β); 3 α ,17,21-Trihydroxy-5 α -pregnane-11,20-dione, Tetrahydrocortisone (3 α ,5 α); 3 α ,17,21-Trihydroxy-5 β -pregnane-11,20-dione, Tetrahydrocortisone (3 α ,5 β); 17,21-Dihydroxy-4-pregnene-3,11,20-trione, Cortisone; 17,21-Dihydroxy-1,4-pregnadiene-3,11,20-trione, Prednisone; 11 β ,21-Dihydroxy-18-aldo-5 α -pregnane-3,20-dione, Dihydroaldosterone (5 α); 3 β ,11 β ,21-Trihydroxy-18-aldo-5 α -pregnan-20-one, Tetrahydroaldosterone (3 β ,5 α); 3 α ,11 β ,17,21-Tetrahydroxy-5 α -pregnan-20-one, Tetrahydrocortisol (3 α ,5 α); 3 α ,11 β ,17,21-Tetrahydroxy-5 β -pregnan-20-one, Tetrahydrocortisol (3 α ,5 β); 3 α ,17,20 β ,21-Tetrahydroxy-5 β -pregnan-11-one, β -Cortolone; 11 β ,17,20 α ,21-Tetrahydroxy-4-pregnen-3-one, 20 α -Dihydrocortisol; 11 β ,17,20 β ,21-Tetrahydroxy-4-pregnen-3-one, 20 β -Dihydrocortisol.

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ways and enzymatic mechanisms. Studies directed toward a better understanding of the mode of action of the steroid hormones will also be considered in some detail.

The fact that metabolic pathways are common to many species, to many tissues, and for several classes of steroid hormones has raised serious problems in the organization of this review. It is intended that the tabular presentation of data will give a broad picture, whereas in the text special aspects have been selected for more detailed discussion.

BIOSYNTHESIS

ADRENALS

The formation in adrenal glands of steroid hormones and intermediates from both steroidal and non-steroidal precursors has been studied in many species.

Human.—Solomon *et al.* (184) separated human fetal adrenals into two fractions: one consists of a "fetal zone" and the other is mainly adult tissue with some "fetal zone" contamination. Both fractions, and particularly the "fetal zone," can carry out the transformation of progesterone to 17-hydroxyprogesterone and to androstenedione. In a study with slices of whole human fetal adrenal glands, Bloch & Benirschke (15) describe the formation from acetic acid-1- C^{14} of dehydroepiandrosterone, androstenedione, and pregnenolone. In a second series of incubations, they isolated by carrier-dilution dehydroepiandrosterone, androstenedione, 11 β -hydroxyandrostenedione, pregnenolone, and cortisol. They conclude from their studies that during the second quarter of gestation predominantly C_{19} steroids are formed. These data are in accord with the observations of Francis *et al.* (67) that dehydroepiandrosterone is an important steroidal constituent of meconium. Human adrenal tissue from fetuses as young as 11 weeks of age could synthesize Porter-Silber chromogens that have the R_F of cortisol, but only tissue from the older fetuses could utilize pregnenolone as a cortisol precursor. This suggests that Δ^5 -3 β -hydroxysteroid dehydrogenase is either unstable or absent (212).

Lombardo & Hudson (128) incubated pregnenolone, progesterone, 17-hydroxyprogesterone, deoxycorticosterone, and 11-deoxycortisol with human adrenal slices or mince and confirmed the presence of 11 β -, 17-, and 21-hydroxylase activity. They further noted that 21-hydroxysteroids did not undergo hydroxylation at C-17. The formation of C^{14} -aldosterone from C^{14} -corticosterone has been reported (143). Incubation of human adrenal slices in the absence of exogenous substrates yielded 20 α - and 20 β -dihydrocortisol (201).

Lombardo *et al.* (129) detected cortisol, cortisone, corticosterone, 17-hydroxyprogesterone, 11-deoxycortisol, and 11 β -hydroxyandrostenedione in human adrenal venous blood. It is interesting that, although dehydroepiandrosterone has been found in peripheral plasma as the sulfate, only in one pa-

tient of 12 studied was a substance having the chromatographic mobility of dehydroepiandrosterone detected in the adrenal venous effluent. Touchstone *et al.* (200) also found 11-deoxycortisol in human adrenal venous blood. The formation of estrone and estradiol from testosterone by slices of adrenal cortical carcinoma tissue from man (9) provides a partial explanation for the high levels of urinary estrogens often observed in patients with this disease.

Other species.—In addition to its function as a female hormone, progesterone occupies a key position in the biosynthesis of the other steroid hormones. Halkerston *et al.* (80) have described a soluble enzyme system, derived from bovine adrenal mitochondria, which requires NADPH_2^5 and oxygen and which converts cholesterol primarily to progesterone. The cholesterol side chain is also cleaved by the soluble fraction of bovine adrenal cortical homogenates and yields isocaproic acid (186). Shimizu *et al.* (181) have obtained a much higher yield of isocaproic acid after the incubation of 20α -hydroxycholesterol with a similar soluble system; this suggests that 20α -hydroxylation may be the first step in the biosynthetic pathway from cholesterol to pregnenolone.

Evidence on the precursors of aldosterone has been recently reviewed by Katzman *et al.* (108). A series of experiments reported by Ayres and co-workers (8) indicates that at least 50 per cent of the aldosterone produced by bovine adrenal capsule strippings is derived from corticosterone. The major pathway is probably progesterone \rightarrow deoxycorticosterone \rightarrow corticosterone \rightarrow aldosterone, although a pathway through progesterone not involving corticosterone may also occur to some extent. The localization of aldosterone biosynthesis in the bovine adrenal zona glomerulosa has been further verified by Stachenko *et al.* (185). Neher & Wettstein (149) have isolated from hog adrenal glands the lactone of $11\beta,18$ -dihydroxy-3-keto-4-etienic acid, which may have been derived from 18 -hydroxycorticosterone, a possible intermediate in aldosterone biosynthesis.

The formation of aldosterone by frog adrenal tissue (203) has also been demonstrated. Results obtained with other species are summarized in Tables I, II, and III (36).

Bovine adrenal homogenates incubated with progesterone- 21-C^{14} produced more corticosterone than cortisol in an isotonic medium than in hypotonic KCl (50). No effect of medium concentration on the relative amounts of the two compounds was observed in similar experiments in which cholesterol- 4-C^{14} was used as substrate. Addition of adrenal medullary homoge-

⁵ In accordance with the newly recommended usage [see *Nature*, 188, 464–66 (November 5, 1960); *Science*, 132, 1548–50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide, reduced form (NADH_2), for diphosphopyridine nucleotide, reduced form (DPNH); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH_2), for triphosphopyridine nucleotide, reduced form (TPNH).—EDITOR.

nate or catecholamines to bovine adrenal cortical homogenates increased the conversion of progesterone to cortisol and corticosterone (38).

The general requirement for NADPH_2 and O_2 and the subcellular localization of the 17-, 21-, and 11 β -hydroxylases in the bovine adrenal are now fairly well established (163). 17-Hydroxylase in the guinea pig adrenal is associated with the microsomal fraction (86). In contrast to the localization of the 21-hydroxylase activity in the microsomes of the bovine adrenal (172), most of this activity in fetal adrenal homogenates in man sedimented at 12,000 g (212). Corticosterone administered to rats or incubated with rat adrenal sections decreased their responsiveness to ACTH, suggesting that blood levels of corticosterone may control its production (158). Staudinger (187) reported that hog adrenal microsomes, NADH_2 , and ascorbic acid together can replace NADPH_2 as cofactor for 11 β -hydroxylation of deoxycorticosterone by ad-

TABLE I
BIOSYNTHESIS OF C_{21} STEROIDS *IN VIVO*

Species	Substrate	Product	Source	Reference
Golden hamster	acetate	cortisol	adrenal vein blood	178
Guinea pig	cholesterol	cortisol	urine	215
Guinea pig	sitosterol	cortisol	urine	216

renal mitochondria. He postulated that the microsomal ascorbic acid-dependent NADH_2 -oxidase (110) produces a hydroxyl radical or activated hydroperoxide which can hydroxylate deoxycorticosterone in the presence of a suitable hydroxylase. However, Tomkins *et al.* (197) succeeded in separating the adrenal 11 β -hydroxylase activity into three enzymic components, which, in addition to NADPH_2 and O_2 , required another unidentified cofactor. Ascorbic acid was inactive in this system. Catalase does not inhibit hydroxylation (172). This is not conclusive evidence, since it is not known whether steroid hydroperoxides are attacked by this enzyme.

Bloch & Cohen have studied the formation of steroid hormones by normal mouse adrenals and by adrenal tumors in male mice of the same strain (16). They noted that steroid hormone formation by adrenal tumor tissue was less efficient than by normal adrenals and that during the course of transplantation responsiveness to corticotropin diminished. 21-Hydroxylation was reduced, but 11 β -hydroxylation remained at about the same level. The principal products formed by slices of these adrenal tumors were corticosterone and 11 β -hydroxyandrostenedione.

Dehydroepiandrosterone.—The formation of dehydroepiandrosterone is closely connected with the question of an alternative pathway from pregnenolone to androstenedione without the intermediate formation of progesterone (Fig. 1). Cox has pointed out (41) that greater prominence of Reactions 4 and 5 (Fig. 1) may account for the small increase in pregnanediol ex-

cretion compared with the greater increases in pregnanetriol, 5-pregnene-3 β ,17,20 α -triol, dehydroepiandrosterone, and other androgens excreted by certain patients with adrenal hyperplasias, and also for the excessive excretion of the normal metabolite (64) 5-pregnene-3 β ,17,20 α -triol or dehydroepiandrosterone in some patients with adrenocortical tumors (40, 43, 155).

In an effort to clarify this matter, Neher & Wettstein (151) fractionated two-ton quantities of hog adrenal glands and bovine testes in a search for dehydroepiandrosterone and possible precursors. Dehydroepiandrosterone was found in bovine testes but not in hog adrenals, whereas the reverse was true for 17-hydroxypregnenolone. 17-Hydroxyprogesterone, on the other hand, was found in both tissues studied.

Solomon *et al.* (183) have further elucidated the role of 17-hydroxypregnenolone as a precursor of dehydroepiandrosterone in the human; they administered tritiated 17-hydroxypregnenolone both to a patient who excreted large amounts of dehydroepiandrosterone in the urine and to a normal male. The dehydroepiandrosterone, androsterone, 3 α -hydroxy-5 β -androstane-17-one, and 3 β -hydroxy-5 α -androstane-17-one isolated from the urine of the patient were labeled, thus demonstrating conversion of the administered steroid. In the normal subject, however, the levels of radioactivity in the 17-ketosteroids were so low that no clear-cut decision could be made regarding a conversion.

An *in vitro* experiment was performed by Goldstein *et al.* (73), who isolated doubly labeled dehydroepiandrosterone after incubating pregnenolone-H³ and cholesterol-4-C¹⁴ with a homogenate prepared from human adrenal cortical adenoma tissue.

These findings are consistent with the view that there is a pathway for the synthesis of androgens which does not involve progesterone.

OVARY

Relatively few studies have been made with ovarian tissues. Sweat (189) demonstrated formation of progesterone-C¹⁴ from acetate-1-C¹⁴ by bovine ovaries. Duncan *et al.* (48) measured the progesterone content and rate of biosynthesis of swine corpora lutea slices during the luteal phase of the estrous cycle and during pregnancy. The capacity of luteal tissue to synthesize progesterone generally paralleled the morphological development and regression of the corpus luteum and was stimulated by the addition of pregnenolone or NAD. Incubation of normal human ovary slices, however, did not produce detectable amounts of progesterone or 17-hydroxyprogesterone unless gonadotropin was added to the medium (122). Ovaries from patients with Stein-Leventhal syndrome, incubated with or without the addition of pregnenolone or gonadotropin, released only androstenedione into the medium. Anliker *et al.* (2) analyzed a virilizing ovarian tumor for steroids and found testosterone (90 μ g/kg), androstenedione (370 μ g/kg), androsterone (60 μ g/kg), and small amounts of progesterone.

Hollander & Hollander (87) confirmed the conversion of testosterone to

TABLE II
 IN VITRO BIOSYNTHESIS

Species	System	Substrate	Products	Reference
Human (fetus)	adrenal slices	acetate	pregnenolone cortisol dehydroepiandrosterone androstenedione	15
Human (fetus)	adrenal homogenate	pregnenolone progesterone	—	212
	adrenal 12,000 g precipitate	17 α -hydroxyprogesterone 17 α -hydroxyprogesterone	cortisol cortisol cortisol	
Human	adrenal homogenate (normal)	17-hydroxyprogesterone 11-deoxycortisol	cortisol 11-deoxycortisol cortisol	18
	(congenital adrenal hyperplasia)	17-hydroxyprogesterone	— cortisol	
Human	adrenal cortical adenoma homogenate	11-deoxycortisol H ³ -pregnenolone C ¹⁴ -cholesterol	dehydroepiandrosterone	73
Human	adrenal slices or mince	pregnenolone	progesterone 17-hydroxyprogesterone corticosterone cortisol	128
		progesterone	17-hydroxyprogesterone corticosterone cortisol	
		17-hydroxyprogesterone 11-deoxycortisol dehydrocorticosterone	cortisol cortisol corticosterone	
Human	adrenal slices	corticosterone	aldosterone	143
Bovine	adrenal capsule strippings	progesterone	corticosterone aldosterone	8
		deoxycorticosterone	corticosterone aldosterone	
		corticosterone	aldosterone	
Bovine	adrenal slices (a) glomerulosa	progesterone	corticosterone aldosterone	185
		deoxycorticosterone	corticosterone aldosterone	
		corticosterone 11-deoxycortisol 17-hydroxyprogesterone	aldosterone cortisol cortisol	
	(b) Fasciculata-reticularis	progesterone	corticosterone cortisol	
		deoxycorticosterone corticosterone 17-hydroxyprogesterone 11-deoxycortisol	corticosterone — cortisol cortisol	
Bovine	adrenal homogenate	progesterone	16 α -hydroxyprogesterone 3 β ,16 α -dihydroxy-5 α -pregnan-20-one	217
Bovine	adrenal mitochondria extract	cholesterol	progesterone	80
Golden hamster	adrenal homogenate	squalene	cortisone dehydrocorticosterone cortisone	178
		cholesterol	dehydrocorticosterone cortisol corticosterone cortisol cortisone	
	adrenal slices	4-cholesten-3-one		

TABLE II (continued)

Species	System	Substrate	Products	Reference
		pregnenolone	corticosterone dehydrocorticosterone cortisol cortisone corticosterone cortisol cortisone 11-deoxycortisol cortisol cortisone corticosterone dehydrocorticosterone corticosterone cortisol cortisone 17-hydroxyprogesterone deoxycorticosterone dehydrocorticosterone cortisol cortisone (trace)	
		17-hydroxypregnenolone		
		progesterone		
		11 β -hydroxyprogesterone		
		17-hydroxyprogesterone		
		deoxycorticosterone		
		11-deoxycortisol		
Guinea pig	adrenal microsomes	pregnenolone or progesterone	deoxycorticosterone 11-deoxycortisol	86
Rat, mouse	testicular homogenate	progesterone	deoxycorticosterone	45
Mouse	testicular interstitial cell tumor	progesterone	deoxycorticosterone	
Bullfrog	adrenal mince	progesterone	aldosterone	203
Human	minced ovarian stroma or corpus luteum	progesterone	6 β -hydroxyprogesterone pregnanedione 5 α -pregnanedione 20 α -dihydroprogesterone 20 β -dihydroprogesterone 20 α -hydroxypregnan-3-one 17-hydroxyprogesterone 17,20 α -dihydroxy-4-pregnen-3-one androstenedione estrone estradiol-17 β	189
Human	ovary slices: normal	17-hydroxyprogesterone	17,20 β -dihydroxy-4-pregnen-3-one	173
Bovine	corpus luteum mince	acetate	progesterone	189
Human	testicular tumor slices	acetate	17-hydroxyprogesterone dehydroepiandrosterone androstenedione adrenosterone 11 β -hydroxyandrostenedione testosterone 17-hydroxyprogesterone testosterone androstenedione 11 β -hydroxyandrostenedione androstenedione 11 β -hydroxyandrostenedione 11 β -hydroxytestosterone adrenosterone	145
		progesterone		
		testosterone		
Human	testicular tumor transplant slices	acetate	progesterone testosterone	223
Horse	testis perfusion	acetate	progesterone 17-hydroxyprogesterone androstenedione estrone estradiol	153 176

TABLE III

STEROIDS ISOLATED FROM TISSUES AND BODY FLUIDS

Source	Steroid	Reference
Human adrenal vein blood (mammary carcinoma)	cortisol; corticosterone; 11-deoxycortisol; 17-hydroxyprogesterone; 11 β -hydroxyandrostenedione	129
Human adrenal vein blood	11-deoxycortisol	200
Human pheochromocytoma	cortisol	144
Incubated human adrenal slices	20 α -dihydrocortisol; 20 β -dihydrocortisol	201
Human adrenal-adrenogenital syndrome	progesterone	229
Human adrenal-congenital adrenal hyperplasia	17-hydroxyprogesterone	18
Human adrenal slices, incubated, Conn's syndrome; Cushing's syndrome; gonadal dysgenesis	cortisol; corticosterone; aldosterone	10
Human adrenal slices, incubated, hypertension; cancers	cortisol; corticosterone; 11 β -hydroxyandrostenedione	39
Human adrenal slices, incubated, adrenocortical hyperfunction	cortisol; corticosterone; cortisone; 11 β -hydroxyandrostenedione; aldosterone	49
Hog adrenal	3 β -hydroxy-5 α -androstane-11,17-dione; 3 α ,17,21-trihydroxy-5 α -pregnane-11,20-dione	213
Hog adrenal	3 β ,16 α -dihydroxy-5 α -pregnan-20-one	147
Hog adrenal	3 α ,16 α -dihydroxy-5 α -pregnan-20-one	148
Hog adrenal	16 α -hydroxyprogesterone	217
Hog adrenal	20 α -hydroxy-4-pregnen-3-one; 17,20 α -dihydroxy-4-pregnen-3-one; 11 β ,20 α -dihydroxy-4-pregnen-3-one; 11 β ,17 α ,20 β -trihydroxy-4-pregnen-3-one; 17,20 α ,21-trihydroxy-4-pregnen-3-one; 17,20 β -dihydroxy-4-pregnene-3,11-dione; 17,20 α -dihydroxy-4-pregnene-3,11-dione; 11 β ,17 α -dihydroxy-4-androsten-3-one; 3 α ,11 β -dihydroxyandrostane-17-one	150
Hog adrenal	11 β -hydroxyprogesterone; 11-ketoprogesterone; 17-is aldosterone; lactone of 11 β ,18-dihydroxy-3-keto-4-etiolic acid	149
Hog adrenal	pregnenolone; 17-hydroxypregnenolone	151
Dog adrenal vein blood (after ACTH)	17-hydroxypregnenolone	35
Golden hamster adrenal vein blood	cortisol	177
Adrenal vein blood of young calf	20 α -dihydroprogesterone	11
Incubated bullfrog adrenal	aldosterone; corticosterone	34
Incubated interrenal tissue a) <i>Anoplopoma fimbria</i> (sablefish) b) <i>Mugil cephalus</i> (mullet) c) <i>Tilapia mossambica</i> (cichlid)	cortisol cortisone; cortisol corticosterone	146
<i>Oncorhynchus tshawytscha</i> plasma (Pacific salmon); rainbow trout plasma	cortisol; cortisone	82
Plasma of male spawning <i>Oncorhynchus nerka</i> (Sockeye salmon)	cortisol; cortisone; aldosterone; corticosterone	162
Plasma of Sockeye salmon	cortisone; cortisol; corticosterone; 17-hydroxyprogesterone	101, 102
Plasma of Pacific salmon	17,20 β -dihydroxy-4-pregnen-3-one	100
Human ovary	17-hydroxyprogesterone; androstenedione	228

TABLE III (continued)

Source	Steroid	Reference
Human placenta; ovary; pregnancy blood; pregnancy fat (not uterus)	progesterone; 20 α -dihydroprogesterone; 20 β -dihydroprogesterone	230
Human pregnancy plasma	estrone; estradiol-17 β ; estriol	154
Human pregnancy bile	estrone; estradiol-17 β ; estriol; 16-epiestriol	1
Human placenta	16-ketoestradiol	44
Bovine corpora lutea (not placenta, uterus vein blood, adrenal, fetal testis)	progesterone; 20 β -dihydroprogesterone	74, 76, 119
Pregnant rat ovary; blood; fat (not fetus, placenta, uterus, amniotic fluid)	progesterone; 20 α -dihydroprogesterone	219
Bovine placenta	estrone; estradiol-17 β ; estradiol-17 α	75
<i>Squalus suckleyi</i> (dogfish) ovaries	estrone; estradiol-17 β	222
<i>Protopterus annectens</i> (lungfish) ovaries	progesterone; estrone; estradiol-17 β ; estriol	42
Starfish (<i>Pisaster ochraceus</i>) ovaries	estradiol-17 β	19
Hog testis	dehydroepiandrosterone	151

estradiol in slices of dog ovary and found some stimulation of the conversion in the presence of follicle-stimulating hormone administered either *in vivo* or *in vitro*. In similar studies with mouse ovaries (88), they failed to detect any correlation between estrogen synthesis from testosterone and the incidence of spontaneous mammary cancer in pure strain mice.

TESTIS

Perfusion of the testis of a gonadotropin-stimulated stallion with acetate-1- C^{14} led to the formation of progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone (176), as well as estrone and estradiol-17 β (153). The formation of estrone and estradiol-17 β from testosterone was also demonstrated with stallion testis mince (9).

An unexpected observation was that of Dominguez *et al.* (45), who found that progesterone- C^{14} was converted in small amounts to deoxycorticos-

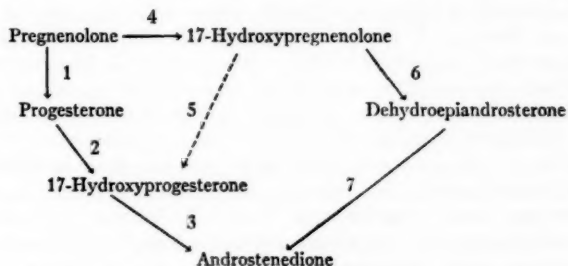


FIG. 1. Postulated pathways for androgen biosynthesis.

terone- C^{14} by homogenates of normal rat and mouse testis. This finding served to clarify their observation that some interstitial cell tumors of the testis in mice subjected to prolonged estrogen treatment produce deoxycorticosterone.

In an extensive study of the clinical and biochemical features of a human interstitial cell tumor of the testis, Savard *et al.* (175) described the incubation of this tissue with radioactive acetate, testosterone, and progesterone. From the incubation with acetate, radioactive testosterone, androstenedione, 11β -hydroxyandrostenedione, adrenosterone, dehydroepiandrosterone, and 17 -hydroxyprogesterone were isolated. The results of the incubations with testosterone and progesterone were in accord with these findings, as were the preoperative urinary excretion patterns in the patient, who had elevated urinary levels of androsterone, 3α -hydroxy- 5β -androstan- 17 -one, dehydroepiandrosterone, and 3α , 11β -dihydroxy- 5α -androstan- 17 -one. These observations form an interesting parallel with the findings in the rodent tumors.

Wotiz (223) studied steroid hormone production by a human embryonal carcinoma of the testis transplanted to the hamster cheek pouch. This tumor produced testosterone.

PLACENTA

Using the placental microsome system of Ryan (170), Longchampt *et al.* (130) demonstrated that 19 -hydroxyandrostenedione serves as an intermediate in the conversion of $C_{19}O_2$ steroids to estrogens in this tissue.

TRANSFORMATIONS OF STEROID HORMONES

Insofar as it is possible, the subject of transformations of steroid hormones by isolated tissues and enzymes extracted therefrom will be discussed in terms of the chemical reactions involved. *In vivo* experiments will also be cited.

Δ^4 -REDUCTASES

The first, and probably the rate-limiting step in reduction of the A ring of the neutral steroid hormones (194) is the irreversible reduction of the $4,5$ double bond. McGuire & Tomkins (137, 139, 196) have presented evidence that Δ^4 - 5α -hydrogenation by rat liver microsomes, like Δ^4 - 5β -hydrogenation by the soluble fraction (195), is catalyzed by a number of enzymes of high substrate specificity, each requiring $NADPH_2$ as hydrogen donor. Solubilization and separation of these enzymes were not achieved, but differential effects on the relative reductase activities for five steroids in individual rats were noted after thyroxine administration, on aging or ethanol treatment of the microsomes, or on inhibition by androstane- $3,17$ -dione. These data indicate the multiple nature of the Δ^4 - 5α reductase and that the action of each is probably limited to a single compound. The observation that the reduction of the double bond of cortisol but not that of other corticosteroids is specifically depressed in humans with hepatic cirrhosis (160) suggests independent

functioning of the Δ^4 reductases *in vivo*. Gold (71), however, reported parallel delays in plasma cortisol and 11-deoxycortisol clearance in liver disease and hypothyroidism, which may indicate that the catabolism of both steroids was impaired to the same extent in these disorders.

The rate of reduction of the 4,5 double bond varies in response to a variety of factors. Several groups of investigators (63, 125, 225) have found that liver microsomes from female rats contain substantially more Δ^4 -5 α reductase activity than those from male rats, whereas the soluble Δ^4 -5 β reductases, quantitatively less important, occur only in the livers of male rats (63, 225). Ovariectomy or estrogen treatment have little effect (63, 77, 225), but testosterone treatment decreases and castration increases Δ^4 -5 α reductase activities, especially in young rats (77, 225). These enzyme activities in the male are also depressed by cortisone administration, but the effects of adrenalectomy are not yet clear (77, 206). Since the sex difference is apparent even in rats gonadectomized well before puberty, the primary influences on Δ^4 reductase levels are probably non-gonadal (225). Hypophysectomy dramatically decreased Δ^4 -5 α reductase activity (63), and thyroxine administration markedly stimulated reduction of Δ^4 -3-ketosteroids, both by making more NADPH available in rat liver homogenates and by increasing the absolute Δ^4 -5 α reductase activities (138). Hepatic Δ^4 -hydrogenation of corticosterone in both male and female rats was reported to be an almost linear function of food intake (83).

Some species differences in the ability of liver homogenates to reduce the 4,5 double bond have been recently summarized (29, 46). In the golden hamster the males have larger adrenal glands than the females. Liver tissue from the males reduces Ring A more rapidly than that of the females. Thus, in both rat and hamster there is a positive correlation between Ring A reduction in the liver and adrenal weight. In the guinea pig, however, only the adrenal gland contains significant Δ^4 reductase activity, whereas several other species have more limited adrenal Δ^4 reductase activities (29).

Hydroxysteroid dehydrogenases and ketosteroid reductases.—The term dehydrogenase will be used here to designate an enzyme which catalyzes a freely reversible reaction, and reductase will designate an apparently irreversible reduction of a carbonyl group.

Studies on the 3 α - and 3 β -hydroxysteroid dehydrogenases have not revealed as clear-cut subcellular localization nor high substrate and coenzyme specificity as have the Δ^4 reductases. Hurlock & Talalay (98) have described a microsomal 3 α -hydroxysteroid dehydrogenase system in male rat liver which, like the soluble system partially purified by Tomkins from the same source (194, 198), utilizes NADPH₂ or NADH₂ as hydrogen donor.

Rubin (169) demonstrated that when androstane-3,17-dione and androstenedione were incubated with rat liver homogenates, reduction to 3 α - and 3 β -hydroxysteroids occurred. The ratio of the reduction rates of 3-keto compounds to 3 β - and 3 α -hydroxy compounds depends upon the sex of the animal; males have about a twentyfold higher ratio than females. Castration of

TABLE IV
 CATABOLISM OF C-21 STEROIDS *IN VITRO*

Species	System	Substrate	Products	Reference
Human	liver (post mortem) homogenate	progesterone	5 β -pregnane-3,20-dione; 5 α -pregnane-3,20-dione; 3 α -hydroxy-5 α -pregnan-20-one; 3 β -hydroxy-5 α -pregnan-20-one (trace); 3 α -hydroxy-5 β -pregnan-20-one; 5 β -pregnane-3 α ,20 α -diol	3
Human	uterine fibroblasts, tissue culture	progesterone cortisol	20 β -dihydroprogesterone; 20 α -dihydroprogesterone; 5 α -pregnane-3,20-dione 20 β -dihydrocortisol; 20 β -dihydrocortisone	190
Human	placenta 105,000 g	progesterone	20 α -dihydroprogesterone	126
Mouse	areolar tissue (fibroblasts)	cortisol	20 α -dihydrocortisol; dihydrocortisol (5 β); cortisone; corticosterone; 11 β -hydroxy-4-androstene-3,17-dione	12
Rat	liver slices or homogenate	progesterone or 16 α -hydroxyprogesterone	3 β , 16 α -dihydroxy-5 α -pregnan-20-one; 3 α , 16 α -dihydroxypregnan-20-one; 3 α , 16 α -dihydroxy-5 α -pregnan-20-one	217
Rat	liver homogenate plus NADPH ₂	aldosterone	dihydroaldosterone (5 α); tetrahydroaldosterone (3 β , 5 α)	157
Rat	liver perfusion	prednisone	prednisolone; cortisol; cortisone; 20 β -dihydroprednisone	180
Rat	liver homogenate	prednisolone	cortisol; 20 β -dihydrocortisol; tetrahydrocortisol (3 α , 5 α)	208
Rabbit	liver homogenate	deoxycorticosterone	20 α -dihydrodeoxycorticosterone; 20 β -dihydrodeoxycorticosterone; 3 α , 21-dihydroxy-5 α -pregnan-20-one; 3 β , 21-dihydroxy-5 α -pregnan-20-one; 3 α , 21-dihydroxy-5 β -pregnan-20-one	192

the male produced a lower ratio which could be returned to normal by androgen administration. The 3 β to 3 α ratio in gonadectomized females could be raised with androgens. The livers of male guinea pigs have more 3 β - than 3 α -hydroxysteroid dehydrogenase (81).

6 ξ -Hydroxysteroid dehydrogenase activity was found in rat liver tissue by Breuer *et al.* (27), who showed interconversion of 6 ξ -hydroxyestradiol and 6-ketoestradiol. Curiously, the reduction of 6-ketoestrone was irreversible.

Many tissues are capable of interconverting the 11-carbonyl and 11 β -hydroxyl of corticosteroids (Table IV); in the rat the most active are liver and kidney (132, 133). Oxidation of the 11 β -hydroxyl predominates in rat kidney (132, 133) and probably in peripheral tissues generally (32). Human synovial tissue, however, is incapable of reducing cortisone to cortisol (161). The microsomal 11 β -hydroxysteroid dehydrogenases of rat, guinea pig, or bovine liver catalyze readily reversible reactions with either NAD or NADP as hydrogen acceptors (98). The human placental enzyme activity has low substrate specificity and dual pyridine nucleotide specificity (156). Bush & Mahesh (32) observed that administered 11-ketosteroids with a *cis* A/B ring fusion were not reduced to 11 β -hydroxysteroids, whereas those with a *trans* A/B ring fusion were readily reduced. The greater ease with which the 11-keto-5 α metabolites are reduced probably largely accounts for the predominance in human urine of 11 β -hydroxy-5 α steroids over 11-keto-5 α steroids

and for the reverse situation with corresponding 5β steroids (see also 20, 174). The 2α -methyl 11-oxygenated steroids resist oxidation or reduction at C-11, suggesting 11β -hydroxysteroid dehydrogenase interaction with the α side of steroid substrates (33). This postulated enzyme-substrate interaction may be the basis of the limited interconversion of tetrahydrocortisol ($3\alpha,5\beta$) and tetrahydrocortisone ($3\alpha,5\beta$) in man (72), in contrast to the substantial conversion of tetrahydrocortisone ($3\alpha,5\alpha$) to tetrahydrocortisol ($3\alpha,5\alpha$) (32). Prolonged cortisol treatment in the mouse markedly increased the ability of the thymus to transform cortisol to cortisone, but only slightly increased this ability in lymph nodes, and decreased it in the spleen (47). Two 17β -ol dehydrogenases which attack testosterone have been isolated from guinea pig liver and kidney (52, 78, 112, 116, 211). The enzyme concentrated in the soluble fraction requires NADP as cofactor, whereas the one in the mitochondria requires NAD.

Evidence for enzymes capable of oxidizing 16-hydroxy groups comes from the work of Levitz *et al.* (124), who injected C^{14} -estriol into humans and recovered 16-ketoestradiol and 16-epiestriol from the urine. Breuer (21) found weak 16α -hydroxysteroid dehydrogenase in rabbit liver, and Ryan (171) found reduction of 16-ketoestrone to 16-epiestriol and estriol by human placental extracts.

Substrate specificity studies on the placental estradiol- 17β -dehydrogenase have been reported (120). This enzyme does not utilize non-aromatic steroids but attacks a wide variety of aromatic steroids related to the natural estrogens. A similar enzymatic activity was found in rat blood (224) and in the erythrocytes of a number of mammalian and avian species (131, 164). Axelrod & Werthessen (6, 7) report that cow blood oxidizes estradiol- 17β to estrone, whereas estrone is reduced primarily to estradiol- 17α in this fluid (7). The reduction of estrone to estradiol- 17β has been accomplished with bovine cell cultures grown in a blood-free medium (57, 207).

In a series of ingenious experiments employing doubly labeled estrone and estradiol, Fishman *et al.* showed that estrone serves as the principal substrate for the hydroxylation at C-16 (58, 59) as well as in Ring A. The data indicate that any equilibrium between estrone and estradiol lies far on the side of estrone. This suggests that in peripheral tissues estrone may be a more important compound than estradiol.

Enzymes that catalyze the reduction of the corticoid 20-carbonyl to either the 20α - or 20β -hydroxyl are also widely distributed in mammalian tissues (12, 132, 133, 190); highest activities again are in the liver and kidney (43, 133). The subcellular localization and substrate and pyridine nucleotide specificities of the extrahepatic enzymes generally have not yet been definitely determined. Dehydrogenation of the 20-hydroxyl has not been observed *in vitro*, although a very small conversion of β -cortolone to tetrahydrocortisone and tetrahydrocortisol may occur *in vivo* (72).

Reduction of the side chain of cortisone by rat liver homogenates was found to be more rapid in the male (77, 125, 202). Castration depressed side-

chain reducing activity, whereas testosterone administration, if initiated immediately after castration, prevented this change. Ovariectomy only slightly increased the rate of side-chain reduction, whereas estradiol treatment of the male depressed this activity. Cortisone acetate administration reduced the rate of reduction of the C-20 carbonyl in both sexes (77, 202).

Reduction products of progesterone have been isolated from various tissues (Table III). While 20 β -hydroxy-4-pregnene-3-one has been isolated from bovine corpora lutea (74, 76, 119), only the 20 α -epimer occurs in rat ovary, blood, and fat (219). Both isomers are present in human placenta, ovary, and in the plasma and adipose tissue of pregnant women (230), and both are formed from progesterone by incubation with ovarian mince (189). Wiest (218) has partially purified a 20 α -hydroxysteroid dehydrogenase from rat ovary which catalyzes a reversible reaction and is specific for progesterone or 17-hydroxyprogesterone as substrate and NADPH₂ as hydrogen donor. Levels of enzyme activity appear to be inversely related to progesterone secretion and may respond to estrogen or follicle-stimulating hormone. Activity is high during follicular maturation and low in pseudopregnant rats during the development of massive deciduomata (218).

A noteworthy achievement was the crystallization of a 20 β -hydroxysteroid dehydrogenase derived from the mycelium of a *Streptomyces* adapted to 11-deoxycortisol (95, 96). The enzyme was crystallized in 12 per cent yield by a relatively simple procedure which consisted of ultrasonic disruption of the cells, centrifugation, precipitation with ammonium sulfate, adsorption on and elution from calcium phosphate gel, chromatography on diethylaminoethyl cellulose, and, finally, crystallization by the addition of ammonium sulfate.

The isolation of 2-methoxyestrone (54, 117, 118) and of 2-methoxyestradiol (61) from the urine of patients who had received C¹⁴-estrogens was soon followed by the identification of 2-methoxyestradiol in pregnancy urine (68). The 2-hydroxy compounds are probably intermediates. This was suggested by an *in vivo* experiment (5), in which administered 2-hydroxyestradiol was converted in small yield to 2-methoxyestrone, and by the isolation of 2-hydroxyestrone from the urine of a patient who had been given C¹⁴-estradiol (60). The conversion of 2-hydroxyestradiol to 2-methoxyestradiol by guinea pig tissues (4) and by human liver (24) has been reported.

6-HYDROXYLATION

6 β -Hydroxylation of corticosteroids is an important catabolic pathway in the guinea pig (30, 31). 6 β -Hydroxyprogesterone was formed when progesterone was incubated with human ovary (189). Ulstrom *et al.* (204, 205) have observed that 6 β -hydroxycortisol is a major urinary metabolite of cortisol in the newborn infant.

In an extensive series of experiments, Breuer *et al.* (27, 28) found that rat liver tissue oxidized estrone, estradiol, and estriol (25) to 6-hydroxylated derivatives, the configurations of which have not yet been determined.

7-HYDROXYLATION

The isolation of $3\beta,7\alpha$ -dihydroxy-5-androsten-17-one and $3\beta,7\alpha,16\alpha$ -trihydroxy-5-androsten-17-one from the urine of a patient with adrenal cortical carcinoma demonstrates the presence of 7 α -hydroxylase in the human (155). There is as yet no evidence concerning the location of this enzyme system.

16 α -HYDROXYLATION

16 α -Hydroxylated derivatives of pregnenolone and progesterone have been found in several species (Table III) (65, 148). Progesterone is converted to 3 α - and $3\beta,16\alpha$ -dihydroxy-5 α -pregnan-20-one and 3 $\alpha,16\alpha$ -dihydroxy-5 β -pregnan-20-one by rat liver homogenates. The 3β compound is also formed from progesterone by bovine adrenal homogenates and is accompanied by 16 α -hydroxyprogesterone. All four compounds are formed in small amounts (217).

Urinary neutral steroids which possess 16-hydroxy substituents have been known for some time (84, 85, 135). Recently, new representatives of this class of compounds have been isolated. The isolation of 3 $\alpha,16\alpha$ -dihydroxy-5 β -pregnan-20-one from the urine of patients with adrenogenital syndrome of the salt-losing type (148) is particularly noteworthy, since this steroid causes sodium loss in adrenalectomized rats under suitable experimental conditions. Fotherby (64) recovered 5-androstene- $3\beta,16\beta,17\beta$ -triol from normal male urine and later extracted 5-pregnene- $3\beta,16\alpha,20\alpha$ -triol from the same source (65). In addition, Okada *et al.* (155) found $3\beta,16\alpha$ -dihydroxy-5-androsten-17-one, $3\beta,7\alpha,16\alpha$ -trihydroxy-5-androsten-17-one, and $3\beta,16\alpha$ -dihydroxy-5-androstene-7,17-dione in the urine of a patient with adrenal cortical carcinoma. The first of the substances had previously been isolated from the urine of normal males (64, 66).

The formation of estriol from estradiol by human fetal liver has been reported (55). Although this substance had long been thought to be characteristic of the human species, its formation by rat (79) and chicken (99, 140) liver tissue demonstrates a far wider distribution.

In human liver, Breuer & Knuppen (23) found that the main metabolites of 16-ketoestrone are 16-ketoestradiol-17 β and 16 β -hydroxyestrone, whereas estriol, 16-epiestriol, 16 α -hydroxyestrone, and 17-epiestriol were minor transformation products. Breuer & Nocke (26) reported the formation of estriol-3,16 $\beta,17\alpha$ from 16 β -hydroxyestrone by normal liver slices in man. With the isolation of 1,3,5(10)-estratriene-3,16 $\beta,17\alpha$ -triol (26) and 1,3,5(10)-estratriene-3,16 $\alpha,17\alpha$ -triol (22), the roster of the stereoisomers of estriol is now complete. Levitz *et al.* (123) demonstrated the conversion of C¹⁴-labeled 16-ketoestradiol-17 β to estriol and 16-epiestriol.

18-HYDROXYLATION

Although this reaction had previously been shown to occur only in the biosynthesis of aldosterone, 18-hydroxyestrone (127) has now been found as a constituent of human pregnancy urine.

Δ^4 -3-KETOSTEROID ISOMERASE

An outstanding contribution was the crystallization by Kawahara & Talalay (109) of an induced enzyme derived from *Pseudomonas testosteroni* which catalyzes the isomerization of Δ^4 -3-ketosteroids to the conjugated Δ^4 -3-ketosteroid system. The enzyme was purified by a series of precipitation steps and obtained in a yield of 25 per cent and a 3000-fold purification. The reaction catalyzed is intramolecular; no deuterium appears in the product when the reaction is carried out in 98 per cent D_2O .

SIDE-CHAIN CLEAVAGE

No really effective *in vitro* systems in nonendocrine tissues have been found to catalyze the elimination of the side chains of C_{21} -17-hydroxysteroids and thus account for the conversion of such substances to 17-ketosteroids. The microorganisms of the intestinal tract have now been implicated by the work of Wade *et al.* (214). They found that rectal infusions of cortisol produced a hundredfold increase in the levels of 11β -hydroxyetiocholanolone in the urine and a 25-fold increase in the level of 11β -hydroxyandrosterone in the urine, whereas oral cortisol had little effect upon the urinary 17-ketosteroid excretion. The increased excretion of the 11-oxygenated 17-ketosteroids did not occur in patients whose intestinal tracts had been sterilized by the administration of neomycin.

STEROID SULFATE SYNTHESIS

The mechanism of sulfate ester formation has recently been elucidated. A sulfate-activating enzyme system synthesizes adenosine-3'-phosphate-5'-phosphosulfate (165, 166), and then sulfokinases transfer the sulfate from the phosphosulfate to receptor substrates. Nose & Lipmann (152) have partially separated several steroid sulfokinases of different substrate specificity from rabbit and lamb liver, and they observed that steroid sulfokinases are found only in the liver. Greater sulfate formation with 3β -hydroxysteroids and with A/B *trans* steroids found *in vitro* (152, 179) correlates well with *in vivo* results (104).

EFFECTS OF STEROID HORMONES

A phase of the biochemistry of steroid hormones which is attracting increasing attention is designated, rather ambitiously, as studies of the mechanism of action of the hormones or, perhaps more realistically, as studies of the effects of steroid hormones at the molecular level. The gap between phenomena in the whole animal and at the molecular level has not yet been bridged.

CORTICOSTEROIDS

Relatively few experiments on *in vitro* effects of corticosteroids can be related to their metabolic effects. Jensen (105) has shown that NADP oxidase of heart sarcosomes is inhibited by corticosterone at a concentration of

10^{-4} M. Similarly, cortisol at concentrations of 10^{-8} to 5×10^{-4} M has been shown to inhibit oxidative metabolism by liver mitochondria by increasing mitochondrial permeability (69). Blecher & White (13, 14) have suggested that, in cell-free preparations of rat lymphosarcoma, deoxycorticosterone and cortisol affect reactions which regulate the supply of available ATP. Deoxycorticosterone added *in vitro* at a concentration of about 10^{-4} M releases the latent adenine triphosphatase of lymphosarcoma mitochondria. Engel & Scott (56) have shown a small stimulatory effect of 10^{-8} to 10^{-6} M corticosterone on the oxidation of glutamate by glutamic dehydrogenase under conditions that make NAD the rate-limiting reactant. Cortisol had a similar effect at a higher concentration, and epicorticosterone had no effect. An inhibitory effect of a similar order of magnitude was found for the amination of α -ketoglutarate in the presence of NADH_2 .

Continuing earlier work on the effects of corticosteroid administration on enzyme induction in rat liver (111), Civen & Knox (37) demonstrated an increase in liver tryptophan- α -ketoglutarate transaminase within 5 hr. of the administration of 15 mg/kg cortisol. Hepatic levels of glutamic-pyruvic transaminase were increased five- to sevenfold by corticosteroid administration, as well as by fasting, diabetes, and a high protein intake, all conditions under which gluconeogenic activity is enhanced (168). The activity of glutamic-oxalacetic transaminase increases only slightly after corticosteroid administration (167).

PROGESTERONE

Pesch & Topper (159) reported that a number of steroids, among them progesterone, testosterone, and androsterone, at concentrations of 5×10^{-6} M, stimulate C^{14}O_2 production from galactose-1- C^{14} by rabbit liver slices. This effect was at first thought to be on the epimerase reaction (182), but it now appears that aldehyde dehydrogenase is inhibited (199), thus lowering the level of NADH_2 . Isselbacher (103) believes that progesterone exerts its effect by stoichiometrically regenerating NADPH_2 during the course of reduction of the 4,5 double bond. This would not, however, explain the equal effectiveness of androsterone.

ANDROGENS

Similar model systems have not yet been evolved for androgens, although Hurlock & Talalay (97) have found a transhydrogenase in rat liver which is stimulated by 3α -hydroxysteroids. However, since 3α -hydroxy- 5β -androstane-17-one, a non-androgenic steroid, is as effective in mediating transhydrogenation as is androsterone, an androgen, the relation of this effect to androgenicity is far from clear. Stein & Kaplan (188) have presented evidence that in liver the hydroxysteroid-mediated transhydrogenation probably plays a rather minor role, and Bloom (17) came to somewhat similar conclusions using a different experimental system.

While the work has not yet been translated to *in vitro* systems, the exten-

sive studies of Kochakian and his associates on the effects of castration and androgen administration on the levels of *d*-amino acid oxidase (51), transaminases and glutamic dehydrogenase (114, 115), and muscle growth (113) have given leads which merit further investigation. An observation of very considerable interest is that of Fishman & Lipkind (62), who showed that androgens markedly increased the levels of renal β -glucuronidase in mice. This effect appears to be highly specific, and interesting relations between structure and activity have emerged.

ESTROGENS

The systematic work of Mueller (141, 142) on the biochemical events which occur in the uterus of the estrogen-treated rat has given us a view of some reactions which occur during the early phases of growth; but they have not of themselves led to any *in vitro* system in which the effects of estrogens on specific reactions can be studied.

The work of Vilee and his associates, which began as a description of an NAD-linked isocitrate dehydrogenase that was stimulated by estradiol in human placenta, was extended by Talalay, who found that the reaction sensitive to estradiol was not a dehydrogenase but rather a transhydrogenase. This work, and its areas of agreement and disagreement, has been summarized in five reviews published recently (53, 191, 209, 210, 221). The site of action of the estrogen is upon a transhydrogenase, but there is as yet no agreement between the laboratories as to the mechanism of the reaction. However, the Talalay concept, that estradiol and estrone play a coenzymatic role in the transfer of hydrogen, would seem to be rendered less likely by the clear-cut separation by Hagerman & Vilee (78) of two pyridine nucleotide-linked dehydrogenases from an estradiol-sensitive transhydrogenase. The fact that the three enzymatic activities were separated serves to point up the risk of drawing conclusions concerning identification of multiple enzyme activities with a single protein in relatively unpurified systems. Hollander *et al.* (89, 90, 92) studied the substrate specificity of the estrogen-sensitive transhydrogenase. The pattern of specificity proved to be similar to that of estradiol-17 β dehydrogenase (120, 121), although the enzymatic activities are associated with two different proteins.

Jonas & Hollander (107) developed an assay for transhydrogenase activity, and Hollander (91, 93) showed the presence of estradiol-sensitive transhydrogenase in cancerous and non-cancerous breast tissue; Gaull & Vilee (70) detected a similar enzyme in adenohipophysis.

Other studies on *in vitro* effects of estrogens include those of Mason & Gullekson (136) on inhibition of kynurenine transaminase and muscle phosphorylase-*a* by sulfates of estradiol, diethylstilbestrol, and estrone, and by pregnanediol glucosiduronate at concentrations of 10^{-4} to 10^{-6} *M*, but not by the free steroids. Whether or not these effects are related to the surface activity of these compounds cannot be determined at this time. The effectiveness of the biologically inactive pregnanediol glucosiduronate would

seem to cast doubt upon a physiologic significance for this phenomenon.

Extending his studies on effects of estrogens on *in vitro* systems, Hollander found increased uterine phenol-stimulated NADH₂ oxidase (94) in estrogen-treated rats. This activity was not present prior to treatment, and no effect was noted in animals treated with testosterone, cortisone, or progesterone. It was possible to develop conditions under which phenolic estrogens could replace 2,4-dichlorophenol as activators for this system. However, there was no relation between estrogenic activity and the *in vitro* effects of the compounds (193).

Williams-Ashman *et al.* (220) showed that phenolic estrogens can participate in hydrogen transport by virtue of the action of phenolases. In the presence of potato phenolase, NADH₂ is oxidized more readily when estradiol is present than in its absence. The effectiveness of the compound is determined to some extent by structure, but no clear-cut correlations between catalytic effect in the phenolase reaction and biological activity have yet been developed. A second mechanism involves the action of peroxidase and the participation of a phenoxy radical.

EFFECTS OF VARIOUS STEROIDS ON ENZYMES

Yielding & Tomkins (226) reported that progesterone and deoxycorticosterone inhibit NADH oxidase from a variety of mammalian and microbial sources. The K_i is of the order of 10^{-4} to 10^{-6} . Of the compounds studied, diethylstilbestrol and progesterone have the smallest K_i values, and cortisone has the greatest. It is interesting that tocopherol competitively reverses the steroid inhibition effect on the NADH₂-cytochrome-*c* reductase system of of skeletal muscle and bacteria. In extensions of these studies (227), a direct inhibitory effect of a number of steroids at concentrations of 5×10^{-5} to 10^{-6} M on glutamic dehydrogenase was reported. Diethylstilbestrol was also effective. Again, the least polar compounds exerted the greatest effect. These data call to mind the observations of Munck, who studied the interfacial tension-lowering activity of steroids at a heptane-water interface (145). He found that, of the series of compounds studied, progesterone and diethylstilbestrol were most effective in reducing interfacial tension. At concentrations greater than 10^{-5} M, most of the substances studied formed solid, incompressible surface films. It is possible that this characteristic may be related to the effects observed on enzyme proteins.

Marks & Banks (134) showed a non-competitive inhibition of glucose-6-phosphate dehydrogenase from a number of sources, including rat adrenal glands, by a variety of steroids, such as pregnenolone and dehydroepiandrosterone, at concentrations of 4×10^{-6} to 10^{-7} M. 3β -Hydroxy-5 α -androstane-17-one and 3 α -hydroxy-5-androsten-17-one were as effective as pregnenolone and dehydroepiandrosterone. They speculate that, since pregnenolone is a precursor of steroid hormones and dehydroepiandrosterone is a possible intermediate in androgen production, this effect may play some role in determining the availability of NADPH₂ for hydroxylation reactions in

endocrine tissues. The relation of these findings to the interfacial tension-reducing activity of the steroids is difficult to discern, partly because of the paucity of data on this property.

In studying the effects of steroids upon enzyme systems, it is necessary to employ concentrations which can reasonably be expected to occur physiologically. Jensen & Jacobson (106) used estradiol-17 β -6,7-H³ with a specific activity of 117 to 195 mc/mg, to study the localization of the hormone when it was administered in a physiologically effective dose. They found that the amount of estradiol present in the uterus within 2 hr. after subcutaneous injection of the hormone was 1.5×10^{-4} μ g (ca 10^{-13} mole). It is evident that concentrations of the order of 10^{-4} M are well outside of physiological limits.

In attempting to relate *in vitro* effects of steroids to the *in vivo* hormonal effects, it is advisable to examine the effects, not only of biologically active steroids, but also of closely related biologically inactive steroids. The high degree of structural specificity required for biological activity *in vivo* must have its *in vitro* counterpart.

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BIOSYNTHESIS OF ISOPRENOID COMPOUNDS^{1,2}

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Sir Alexander Todd has recently stated (1): "The unravelling of the actual biosynthetic pathway to the terpenoids and the sterols is one of the outstanding achievements of recent years." It is now possible to write every reaction and name every intermediate in the biosynthesis of cholesterol from acetate with a reasonable degree of certainty. Many of the enzymes involved have been obtained in at least a partially purified form and their properties determined. With respect to the biosynthesis of the other terpenoids, there are still points of detail to be settled, but the broad picture has been outlined. Attention is now being devoted to the more intimate details of the mechanisms involved, to purification and properties of the responsible enzymes, and to factors influencing biosynthesis of isoprenoid compounds.

CHOLESTEROL

The biosynthesis of cholesterol is conveniently separable into three phases, (a) acetate to mevalonate, (b) mevalonate to squalene, and (c) squalene to cholesterol, and is thus divided in this review.

Acetate to mevalonate.—Numerous previous reviews have discussed the incorporation of acetate into cholesterol. Although circumstantial evidence that mevalonic acid is an intermediate between acetate and squalene seemed irrefutable, definite evidence that this is the case has now been furnished by Knauss *et al.* (2) with liver preparations that convert acetate to squalene. Mevalonic acid in the form of dibenzylethylenediammonium salt, hydroxamate, and benzhydrylamide were isolated from reaction mixtures containing acetate-1-C¹⁴ and non-radioactive mevalonic acid. All were found to have the same radioactivity, thus establishing by the simplest and most direct approach that mevalonic acid is an intermediate. The over-all conversion required two enzyme fractions, 40 to 55 per cent and 55 to 80 per cent ammonium sulphate precipitates, microsomes, soluble enzymes, glucose-1-phosphate, and nicotinamide-adenine dinucleotide phosphate (NADP).³ Nico-

¹ The survey of the literature pertaining to this review was concluded in October, 1950.

² The following abbreviations are used: NAD for nicotinamide-adenine dinucleotide; NADPH₂ for nicotinamide-adenine dinucleotide phosphate, reduced form.

³ Editorial note: In accordance with the newly recommended usage [see *Nature*, 188, 464-66 (November 5, 1960); *Science*, 132, 1548-50 (November 25, 1960)], nicotinamide-adenine dinucleotide (NAD) has been substituted for diphosphopyridine nucleotide (DPN); nicotinamide-adenine dinucleotide phosphate (NADP), for triphosphopyridine nucleotide (TPN); and nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH₂), for triphosphopyridine nucleotide, reduced form (TPNH).

tinamide-adenine dinucleotide (NAD) had some activity with the preparations used.

A number of investigations have provided evidence that mevaldic acid, 3-hydroxy-3-methylglutaraldehydic acid, is a precursor of mevalonic acid. For example, mevaldic acid is converted to mevalonic acid by a liver system in which further utilization of mevalonic acid is blocked by preincubation with ribonuclease (3); mevaldic acid is reduced to mevalonic acid by a NADPH₂-dependent system of yeast (4, 5) or by a NAD-dependent animal system (6, 7); the incorporation of acetate-1-C¹⁴ into cholesterol by liver homogenates is diluted by mevaldic acid to the same extent as with mevalonic acid (8); mevaldic acid is converted to cholesterol by liver homogenates to the same extent as mevalonic acid (8); and, finally, mevaldic acid, like mevalonic acid, is converted to squalene by yeast preparations (5). Despite these results, in one study (4, 9) it was not possible to show that free mevaldic acid is an intermediate, since added carrier mevaldic acid was recovered without radioactivity from a yeast system that reduced β -hydroxy- β -methylglutaryl coenzyme A to mevalonic acid.

More recently, Brodie & Porter (10) have been able to carry out the biosynthesis of mevalonic acid from labeled acetate by non-particulate avian and mammalian enzyme systems. β -Hydroxy- β -methylglutaric acid, mevaldic acid, and mevalonic acid were isolated in the form of suitable derivatives and recrystallized to constant activity. The presence of definite radioactivity in mevaldic acid was taken as evidence that the mevaldic acid moiety appears at some stage in the conversion of β -hydroxy- β -methylglutaryl coenzyme A to mevalonic acid. The authors suggest that the initial reduction product of β -hydroxy- β -methylglutaryl coenzyme A is hydrolyzed on an enzyme surface to enzyme-bound mevaldic acid, which is then reduced to mevalonic acid. The appearance of small amounts of radioactivity in isolated mevaldic acid is attributed to a slow loss of mevaldic acid from the enzyme surface. The active mevaldic acid reductase of mammalian liver, previously described by Knauss & Porter (11) and Schlesinger (6), is considered by Brodie & Porter to have the function of converting mevaldic acid lost from the surface of the β -hydroxy- β -methylglutaryl coenzyme A reductase to mevalonic acid.

A detailed paper by Durr & Rudney (12) has now appeared covering their work on the reduction of β -hydroxy- β -methylglutaryl coenzyme A to mevalonic acid by a yeast system. Certain aspects of this work have been briefly noted previously (4, 9). The reduction of β -hydroxy- β -methylglutaryl coenzyme A to mevalonic acid is apparently accomplished by a single enzyme for which they propose the name β -hydroxy- β -methylglutaryl coenzyme A reductase rather than the shorter version, β -hydroxy- β -methylglutaryl reductase (13), since the enzyme actually catalyzes a reductive cleavage of an acylthiol-ester bond. For every mole of β -hydroxy- β -methylglutaryl coenzyme A reduced, one mole of mevalonate is formed, two moles of reduced nicotinamide-adenine dinucleotide phosphate are oxidized (reduced nicotinamide-

adenine dinucleotide is inactive), and one mole of coenzyme A is released. Contrary to Brodie & Porter (10), Durr & Rudney found no radioactivity in mevaldic acid that had been added to the reaction and then reisolated after the conversion of β -hydroxy- β -methylglutaryl- C^{14} coenzyme A to mevalonic acid- C^{14} by the system. It is probably fair to say that specific mevaldic acid reductases exist but that free mevaldic acid is not normally an intermediate in mevalonic acid biosynthesis. β -Hydroxy- β -methylglutaryl coenzyme A reductase was purified about 25-fold from bakers' yeast. Partially purified preparations required the presence of thiols, such as glutathione, cysteine, and pantetheine, for protection, and, as might be expected, the enzyme is inhibited by *p*-hydroxymercuribenzoate. It was also demonstrated that no acyl transfer of β -hydroxy- β -methylglutaric acid from coenzyme A to glutathione prior to reduction occurs, since β -hydroxy- β -methylglutaryl glutathione is not reduced by the enzyme. Similarly, the reduction takes place equally well in Tris or phosphate buffer and is not inhibited by arsenate, which suggests that a phosphotransacetylase reaction is not involved in the reduction. The reduction of β -hydroxy- β -methylglutaryl coenzyme A is irreversible and is not stimulated by Mg^{++} , ATP, or inorganic phosphate.

Mevalonate to squalene.—Groups working with liver (14, 15), yeast (16, 17), and pumpkin seedlings (18) have been in agreement for some time that the first step in the utilization of mevalonic acid is phosphorylation to mevalonic acid-5-phosphate by an enzyme which is now termed mevalonic acid kinase or, simply, mevalonic kinase. The enzyme has been purified from yeast by Tchen (17), from rabbit muscle by Markley & Smallman (15), and from mammalian liver by Levy & Popjak (19).

More recently, the purification and properties of mevalonic kinase from liver have been described in great detail by Levy & Popjak (20). Pork liver, in contrast to rat liver, was found to be a superior source. By a series of purification steps involving the use of protamine (precipitates interfering systems), ammonium sulphate, and calcium phosphate gel, the kinase was purified about 100-fold and yielded a preparation with a specific activity of 1.2 μM of mevalonate phosphorylated/min/mg of protein at 37°. Unless air is excluded from incubations, mevalonic kinase requires activation by cysteine or glutathione. The kinase is inhibited by *p*-chloromercuribenzoate but not by iodoacetamide. The enzyme requires a bivalent ion for activation, and this requirement is satisfied with Mg^{++} , Mn^{++} , and Ca^{++} , less well with Fe^{++} or Co^{++} , but not by Ba^{++} , Cu^{++} , or Zn^{++} . ATP or inosine triphosphate (uncontaminated with ATP) are active as the coenzyme of phosphorylation, but cytidine or guanine triphosphates are inactive. The kinase is specific for the (+) form of mevalonate. None of the following compounds is phosphorylated by the enzyme: mevalonic acid lactone, *cis*-5-hydroxy-3-methylpent-2-enoic acid, 5-hydroxy-3-methylpent-3-enoic acid (*cis* and *trans* mixture), 3-oxobutan-1-ol, or farnesol. Mevalonic kinase is inhibited by farnesoate and by 3,7,11-trimethyl dodecanoate but not by the two anhydro analogues of mevalonate or by 3-oxobutan-1-ol, farnesol, 3-hydroxy-3-methylglutarate,

or isovalerate. Although inhibition of cholesterol biosynthesis in liver homogenates from mevalonate by farnesoate had been previously reported by Wright & Cleland (21), it comes as a surprise that the locus of activity may be the mevalonic kinase stage rather than the sesquiterpene stage of mevalonate utilization.

The second step in the utilization of mevalonic acid involves the phosphorylation of mevalonic acid-5-phosphate to yield mevalonic acid-5-pyrophosphate (14, 18, 22). The responsible enzyme is termed phosphomevalonic acid kinase or, simply, phosphomevalonic kinase (20, 22). This enzyme has been partially purified from bakers' yeast (22) and from pork liver (20). In more extended studies of the purification and properties of phosphomevalonic kinase, Bloch *et al.* (23) have increased the specific activity of the enzyme about fiftyfold by starting with a supernatant of yeast autolysate and employing a series of classical purification procedures. The enzyme has no pronounced pH optimum; the activity remains essentially unchanged between pH 5.5 and 10.0. A divalent ion is required for activity, and Mg^{++} is superior to Co^{++} , Zn^{++} , Fe^{++} , or Mn^{++} , whereas Ca^{++} is inactive. Stoichiometric amounts of ADP are formed in the phosphorylation of mevalonic acid-5-phosphate from ATP.

A third step in the utilization of mevalonic acid involves the concerted dehydration and decarboxylation of mevalonic acid-5-pyrophosphate to yield 3-methyl-3-butenyl-1-pyrophosphate, Δ^3 -isopentenylpyrophosphate, or, simply, isopentenylpyrophosphate (5, 24, 25). This compound has been synthesized by Lynen *et al.* (25) and by Yuan & Bloch (26). Bloch *et al.* (23) have purified the enzyme concerned with the dehydration and decarboxylation 120-fold from yeast autolysate. The optimum pH of the enzyme is between 5.5 and 7.4, and a metal requirement is satisfied with Mg^{++} , Mn^{++} , Fe^{++} and Co^{++} . Stoichiometric amounts of ADP and orthophosphate are formed by the "decarboxylating" enzyme from ATP. ADP and carbon dioxide, the third product of the reaction, are formed at identical rates. The decarboxylation and dehydration of mevalonic acid-5-pyrophosphate is accomplished without uptake of tritium from a T_2O medium, as reported previously (27). A number of possible mechanisms for the reaction have been discussed by Bloch *et al.*, and the previous conclusion that the decarboxylation and dehydration is a "concerted" process is sustained. Whether or not the requirement for ATP indicates that mevalonic acid-5-pyrophosphate is further phosphorylated at position 3 prior to decarboxylation and dehydration has not been established. Such a compound would be expected to be quite unstable and difficult, if not impossible, to isolate. At any rate, the conversion of mevalonic acid-5-pyrophosphate to isopentenylpyrophosphate represents a novel biochemical mechanism and is probably best summarized in Figure 1.

Popjak & Cornforth (28) have proposed the name 5-diphosphomevalonic anhydrodecarboxylase for this enzyme which accomplishes the concerted dehydration and decarboxylation. It would seem that the name may not be quite accurate, since the actual substrate may well be, as indicated, a com-

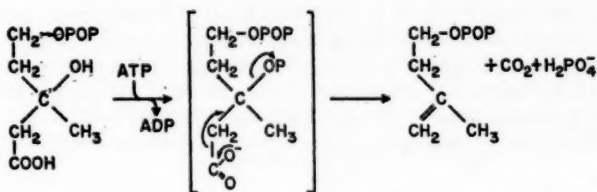
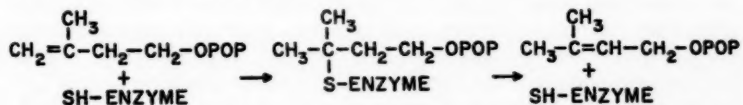


FIG. 1. Postulated mechanism for the concerted decarboxylation and dehydration of mevalonic acid-5-pyrophosphate.

pound containing a phosphate in the 3 position as well as pyrophosphate in the 5 position (the triphosphate). An alternative name not subject to the criticism just raised is "oligophosphomevalonic anhydrodecarboxylase."

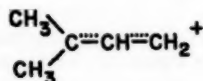
A fourth obligatory step in the utilization of mevalonic acid involves the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate by a recently described enzyme, isopentenyl pyrophosphate isomerase (29, 30). This enzyme has been purified about twentyfold from bakers' yeast. It is also present in rat liver, kidney, and brain, but is apparently absent from heart. At equilibrium, isopentenyl pyrophosphate is about 93 per cent converted to dimethylallyl pyrophosphate, and the reaction is freely reversible. The enzyme has a broad pH optimum, extending from 5.5 to 9.3, and is dependent upon Mg^{++} ions. The enzyme is the only one in the sequence between mevalonic acid and squalene that is sensitive to iodoacetamide. It is also sensitive to *p*-chloromercuribenzoate. Agranoff *et al.* (29) suggest that this sensitivity to iodoacetamide (SH enzyme) may indicate the mechanism for the isomerization shown in Reaction 1.



Cornforth & Popjak considered (31) that the isomerization may equally well be formulated as the electrophilic addition (attack) of a proton to the reactive terminal double bond of isopentenyl pyrophosphate resulting in a cation which is stabilized by the expulsion of a different proton to yield dimethylallyl pyrophosphate. The incorporation of tritium into isopentenol observed after incubation of isomerase with dimethylallyl pyrophosphate was only about 2 per cent that expected from complete equilibrium of the exchangeable positions of isopentenyl pyrophosphate. Agranoff *et al.* (30) suggest that this low value may be attributable to a possible isotope effect, incomplete equilibrium, or to the presence of small amounts of synthetic enzymes concerned with subsequent reactions of the two pyrophosphates.

With respect to subsequent steps in the conversion of mevalonate to squalene, previous ideas concerning mechanisms by which squalene is derived

from C_4 units have required modification, because dimethylallyl pyrophosphate originating from the action of isopentenyl pyrophosphate isomerase on isopentenyl pyrophosphate is involved in squalene biosynthesis (22, 29). Cornforth & Popjak (31) point out that dimethylallyl pyrophosphate has a potentially anionic group in the alpha-position to a double bond and can be expected to lose pyrophosphate ion readily to yield an electron-deficient species



which is partially stabilized by mesomerism between two equivalent forms.



A molecule of isopentenyl pyrophosphate could be attacked by this cation, as described in previous paragraphs for attack by a proton. The product of this attack is geranyl pyrophosphate (Figure 2).

The process can then be repeated with geranyl pyrophosphate attacking isopentenyl pyrophosphate to yield farnesyl pyrophosphate. An enzyme system from yeast or liver, termed "farnesyl synthetase," that accomplishes this condensation has been isolated by Lynen *et al.* (32). Cornforth & Popjak then postulate that farnesyl pyrophosphate condenses with nerolidol pyrophosphate by a mechanism essentially analogous to that involved in the dimethylallyl pyrophosphate-isopentenyl pyrophosphate condensation. Nerolidol pyrophosphate is almost certainly the hydrophilic, acid-labile derivative of nerolidol present in liver enzyme systems incubated with mevalonate (33). They infer the existence of an isomerase that interconverts farnesyl pyrophosphate and nerolidol pyrophosphate (28). The intermediate of the farnesyl pyrophosphate-nerolidol pyrophosphate condensation is then stabilized by the loss, in successive stages, of a proton, the anion x^- (presumably

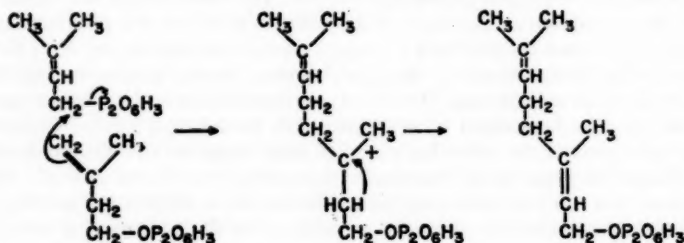


FIG. 2. Postulated mechanism for the biosynthesis of geranyl pyrophosphate from isopentenyl pyrophosphate and dimethylallyl pyrophosphate.

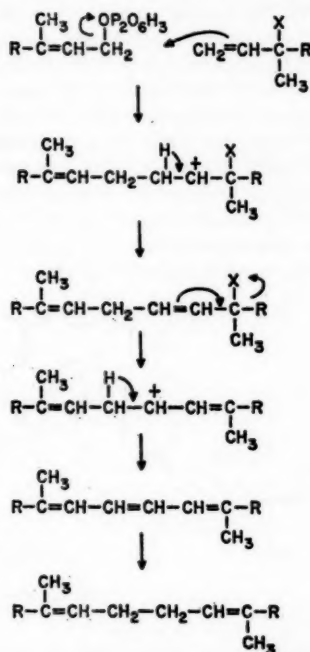


FIG. 3. Postulated mechanism for biosynthesis of squalene from sesquiterpene pyrophosphates.

pyrophosphate), and another proton to yield dehydrosqualene, which is then reduced by a NADPH₂-dependent system to squalene. This mechanism is a slight modification of one originally proposed by Lynen *et al.* (25), which involves both farnesyl pyrophosphate and nerolidol pyrophosphate and a quinone such as coenzyme Q₁₀. The mechanism of Lynen *et al.* is no longer tenable, since it is incompatible with the deuterium uptake experiments of Rilling & Bloch (34). For a more extended discussion of the role of allyl pyrophosphates in squalene biosynthesis, a paper by Goodman & Popjak (35) is recommended.

The mechanism of Cornforth and Popjak has much to recommend it, including agreement with the deuterium uptake experiments of Rilling & Bloch (34; see also 27).

It has been observed by Popjak and collaborators (36, 37) that liver enzyme systems that convert mevalonic acid to cholesterol also accumulate at least 15 "terpenoid acids," including such acids as all-*trans*-farnesoic acid (farnesinic acid), which is an inhibitor rather than an intermediate of cho-

lesterol biosynthesis (21). The suggestion has been made (38) that these acids exert some control over cholesterol biosynthesis, presumably by a feedback mechanism at the mevalonic kinase stage (20). Preliminary studies of the origin of these terpenoid acids from allyl pyrophosphates by liver enzyme systems have been made by Christophe & Popjak (39). The first step in the conversion of the allyl pyrophosphates into terpenoid acids is reversible dephosphorylation into free alcohols by a microsomal phosphatase which has a pH optimum at 7.0 and is inhibited by NaF, phosphate, arsenate, Co^{++} , Mn^{++} , Cu^{++} , Zn^{++} , and bovine serum albumin. Mg^{++} and various chelating agents have no effect on this phosphatase. In a second step, the free alcohols are dehydrogenated to the acids by a soluble enzyme fraction of liver homoge-

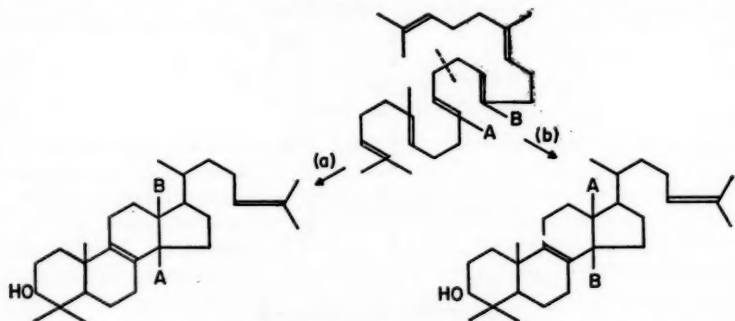


FIG. 4. Possible mechanisms involved in the cyclization of squalene to lanosterol.

nate. This dehydrogenation proceeds best at pH 7.5 and is inhibited by *p*-chloromercuribenzoate, *N*-ethyl maleimide, iodoacetamide, Ag^+ , and Cu^{++} . It is assumed that the aldehydes are intermediary substrates and that alcohol and aldehyde dehydrogenases are involved, together with traces of bound NAD serving as H-carrier. Farnesol is readily oxidized to farnesoic acid by this enzyme system. By following the reduction of added NAD spectrophotometrically, it is observed that more than twice as much NAD is reduced per mole of polyisoprenoid alcohol by this soluble rat liver enzyme system than by crystalline horse liver alcohol dehydrogenase. These polyisoprenoid alcohols are more specific substrates for liver alcohol dehydrogenase than is ethanol, but they do not act as substrates for yeast alcohol dehydrogenase.

Most of the biological experiments involving the use of mevalonic acid have been restricted to the use of the racemic compound because of the unavailability of resolved material (40). In no case has a utilization of mevalonic acid in excess of 50 per cent been recorded, and it has been tacitly assumed that only one enantiomorph, the (+) isomer, is biologically active. R. H. Cornforth *et al.* (41) have now shown in two ways that this is the case. In

one approach, DL-mevalonic acid-2-C¹⁴ was administered to rats and the unutilized form isolated in the form of the benzhydrylamide from the urine. This derivative had a rotation $[\alpha]_D^{24}$ of $+2.2^\circ$ (in ethanol). Wolf *et al.* (42) gave $[\alpha]_D^{24} - 2.0$ (in ethanol) for the diphenylamide of mevalonic acid lactone from distillers' solubles. In a second approach, 5-phosphomevalonic acid was isolated following the action of mevalonic kinase on mevalonic acid, and the optical rotation of the product was determined. The specific rotation was $[\alpha]_D^{24} - 6.1^\circ$ and the molecular rotation $[M]_D^{24} - 13.9^\circ$, thus leaving no doubt as to the stereospecificity of enzymatic reactions involving mevalonic acid.

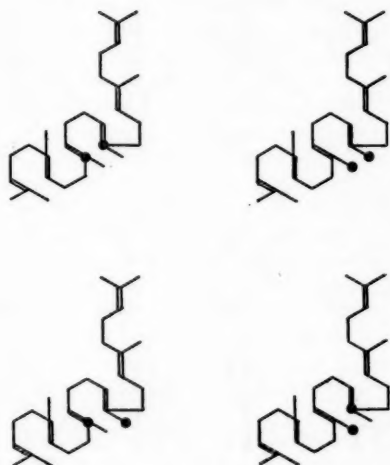


FIG. 5. The four species of squalene used by Maudgal, Tchen & Bloch.

Squalene to cholesterol.—It had been postulated by Eschenmoser *et al.* (43) in 1955 that the cyclization of squalene to lanosterol is a concerted electrophilic addition accompanied by rearrangement and terminated by expulsion of a proton. A feature of this mechanism is the occurrence of two 1:2 methyl shifts, as indicated in Scheme (a) (Figure 4). Alternatively, cyclization could occur with a single 1:3 methyl shift as indicated in Scheme (b). Two groups have now established that the mechanism involving two 1:2 methyl shifts [Scheme (a)] is the one occurring in the cyclization of squalene.

Maudgal, Tchen & Bloch (44) condensed geranyl chloride with a mixture of acetoacetic ester-3-C¹³ and -4-C¹³. The product, geranyl acetone, was then condensed with the triphenyl phosphine derivative of butadiene to give a mixture of four species of squalene-C¹³, as indicated in Figure 5.

This mixture of squalenes was then cyclized to lanosterol by a purified enzyme system involving a minimum of endogenous lanosterol. The lanos-

terol obtained was then degraded by the Kuhn-Roth technique and the resulting acetic acid then converted to ethylene. From the mixture of isotopic squalenes used, several different species of ethylene resulted. It can be shown that, if two 1:2 methyl shifts are involved, one out of 24 molecules of ethylene would contain C^{13} in both carbons, and four out of 24 molecules would contain C^{13} in one carbon. On the other hand, if a single 1:3 methyl shift is involved, no ethylene molecule would contain two C^{13} , and six out of 24 molecules would contain C^{13} in one carbon. Mass spectrographic analysis indicated that ethylene containing C^{13} in both carbons is present to the extent

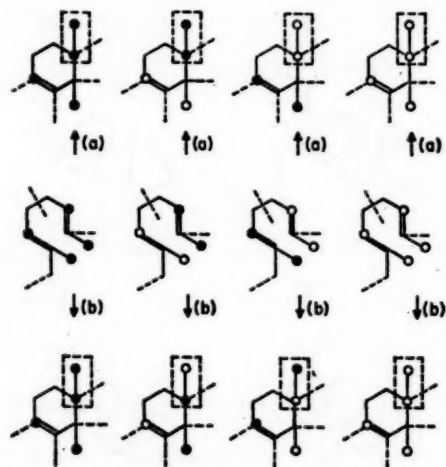


FIG. 6. Distribution of isotope in lanosterol according to alternative mechanisms for the cyclization of squalene.

calculated, and it was concluded that the 1:2 methyl shift mechanism is predominant, if not exclusive, in the cyclization of squalene to lanosterol.

More recently, Cornforth *et al.* (45) prepared mevalonolactone labelled with C^{13} in positions 3 and 4. Biosynthetically, mevalonolactone so labelled should give a mixture of squalenes as indicated in Figure 6. If mechanism (a) (two 1:2 methyl shifts) is operative in the cyclization of squalene, then one half of the acetic acid molecules derived from positions 13 and 18 of cholesterol by the Kuhn-Roth procedure should have both carbon atoms labelled with C^{13} , and the other one-half of the acetic acid molecules should contain no C^{13} . On the other hand, if mechanism (b) (one 1:3 methyl shift) is operative, one-quarter of the acetic acid molecules from positions 13 and 18 should have both carbon atoms labelled with C^{13} , one-half should be singly labelled (methyl or carboxyl), and one-quarter should contain no C^{13} . Actually, the

distribution of acetic acid- C^{13} label derived from cholesterol agreed with that predicted from mechanism (a). Maudgal *et al.* and Cornforth *et al.* are therefore in agreement that in the cyclization of squalene the rearrangement of methyl groups involves two 1:2 shifts.

Largely as a result of studies by Bloch and colleagues, mechanisms and intermediates involved in the conversion of lanosterol to cholesterol have been proposed. A commonly accepted pathway in the rat, summarized by Bloch (46) and Cornforth (47), involves the sequence squalene, lanosterol, 4,4-dimethylcholesta-8,24-dien-3 β -ol, 4,4-dimethylcholesta-8,24-dien-3-one, 4 α -methylcholesta-8,24-dien-3-one, zymosterol, desmosterol, and, finally, cholesterol. Several groups have isolated new intermediates that do not fit into this scheme, which suggests that alternative routes of cholesterol biosynthesis may be of significance.

The existence of a pathway of cholesterol biosynthesis in preputial gland tumor involving the sequence of lanosterol \rightarrow 24,25-dihydrolanosterol \rightarrow 4 α -methyl- Δ^8 -cholestenol \rightarrow Δ^7 -cholestenol \rightarrow 7-dehydrocholesterol \rightarrow cholesterol has been proposed by Kandutsch & Russell (48) following the isolation of the new sterol 4 α -methyl- Δ^8 -cholesten-3 β -ol (49) and kinetic studies of labelled acetate incorporation into various sterols by tumor slices. That this scheme is not restricted to preputial gland tumor follows from the fact that 24,25-dehydrolanosterol, 4 α -methyl- Δ^8 -cholestenol, Δ^7 -cholestenol, and 7-dehydrocholesterol are converted by cell-free homogenates of liver into cholesterol to the extent of 18, 32, 67, and 74 per cent respectively (50).

Wells & Lorah (51) have provided evidence that 4 α -methyl- Δ^7 -cholesten-3 β -ol (methostenol), originally isolated from rat feces (52, 53) and subsequently from rat tissues (54), may be a normal intermediate in cholesterol biosynthesis. Injected sodium acetate-1- C^{14} was rapidly incorporated *in vivo* by the rat into methostenol by combined liver and intestinal tissue and skin. The specific activity of methostenol was 11 and three times that of cholesterol in 5 min. and 3 hr. respectively. Synthetic methostenol-4- C^{14} was quickly absorbed and efficiently converted to cholesterol following oral administration to the rat.

INHIBITORS OF CHOLESTEROL BIOSYNTHESIS

There is a certain rationale to the effect that a compound active as an inhibitor of cholesterol biosynthesis may have utility in the treatment of hypercholesterolemia and atherosclerosis. A number of compounds have been synthesized (some for the first time), evaluated in various *in vitro* and *in vivo* tests, and considered in various reviews; these compounds include α -phenylbutyric acid and α -*p*-biphenylbutyric acid (55, 56); dimethyl acrylic acid, β -hydroxy- β -methyl-glutaric acid, and farnesinic acid (56); 4-methyl mevalonic acid, 2,4-dimethyl mevalonic acid, 3-hydroxy-3-methyl-4-valerolactone, 3-hydroxy-3-methyl-4-butyrolactone, 3-hydroxy-2,3-dimethyl-4-butyrolactone, 3,5-dihydroxy-3,5-dimethyl suberic acid, and certain unsaturated derivatives of this group (57); 2-methyl mevalonic acid, 5-methyl mevalonic

acid, 5-hydroxy-3-methyl-2-pentenoic acid, 5-hydroxy-3-methyl pentanoic acid, 3-hydroxy-3-methyl pentanoic acid, and 3,8-dihydroxy-3,8-dimethyl-decanedioic acid (8); and 2-methyl and 2-ethyl mevalonic acid (58). A number of additional compounds have been studied more recently. Schiffmann & Weiss (59) have compared three compounds, which are related in structure to mevalonic acid, Δ^2 -3-methyl pentenoic acid, Δ^3 -3-methyl pentenoic acid, and 3-methyl-3-hydroxy pentanoic acid, for efficacy in reducing mevalonate incorporation into cholesterol by liver homogenates. The compounds were rated as follows: hydroxy acid $>\Delta^2$ -acid $>\Delta^3$ -acid. Half maximum inhibition of cholesterol formation occurred at concentrations of 5, 15, and 22 $\mu\text{M}/\text{ml}$ respectively.

A new compound, β -hydroxy- β -fluoromethyl- δ -valerolactone (fluoro-mevalonic acid), Δ^1 -testolactone and Δ^4 -androstene-17 α -ol-3-one-17 β -oic acid, as well as sodium fluoride, sodium fluoroacetate, and zinc chloride, have been studied by Singer *et al.* (60) as possible inhibitors of the conversion of acetate to mevalonic acid or cholesterol by liver homogenates. They all inhibited acetate conversion, and the fluoroacetate and zinc chloride inhibited mevalonic acid conversion. The results with fluoromevalonic acid were especially interesting, since inhibition ratios of the order of one for one-half maximum inhibition were obtained. This value is the lowest (most potent inhibitor) yet reported for an analogue of mevalonic acid. Additional studies are awaited with interest. The preparation of α -fluoromevalacetone and γ -fluoromevalolactone have been described by Bergmann & Cohen (61), but biological data have not been reported.

A compound, 1-[p -(β -diethylaminoethoxy)-phenyl]-1-(p -tolyl)-2-(p -chlorophenyl)ethanol, commonly referred to as "MER-29" or, more recently, as "Triparanol," with marked activity against cholesterol biosynthesis and tissue concentration (62 to 66) has now been studied in greater detail with respect to the actual site of inhibition. Avigan *et al.* (67, 68) and Blohm & MacKenzie (69) have shown that the locus of action of this compound is between 24-dehydrocholesterol (desmosterol) and cholesterol. Since desmosterol is present in liver and serum of drug-treated animals (in lieu of cholesterol), it has been suggested that caution be exercised in the use of MER-29 until more is known about the biological properties of desmosterol which has recently been shown by Stokes & Fish (70) to be a normal constituent of rat liver. The work with MER-29 demonstrates that a compound entirely unrelated in structure to any known intermediate in cholesterol biosynthesis may be useful in the control of hypercholesterolemia. Random screening of compounds may be as successful in the search for a practical drug as any procedure based on a rational or antimetabolite approach.

Benzmalecene [N-(1-methyl-2,3-di- p -chlorophenyl propyl)-maleamic acid (α -isomer)] has been shown by Huff & Gilfillan (71) to be a potent inhibitor of the incorporation of mevalonic acid into cholesterol by rat liver homogenates. The inhibition proved to be non-competitive. Oral administration of the compound to normal rats resulted in a significant reduction in plasma

cholesterol. In an accompanying paper, Bergen *et al.* (72) reported that when benzmalecene was administered to patients (six for 10 to 53 days) total serum cholesterol decreased by an average of 18 per cent. There was some decrease in serum alkaline phosphatase and a drop in cholesterol ester levels in proportion to the decrease in serum total cholesterol. Eosinophilia occurred in two subjects. These evidences of toxicity could possibly be circumvented by the use of lower dosage levels of the compound in association with a bile salt-sequestering anion exchange resin MK-135 that shows synergism with benzmalecene in the reduction of plasma cholesterol levels in dogs (73).

Sachs *et al.* (74) have administered benzmalecene to three hyperlipemic and eight normolipemic subjects in doses of 1500 to 2000 mg daily for one to four weeks. Reduction of cholesterol levels occurred in all subjects, averaging 28 per cent with a range of 15 to 46 per cent. Associated with the decrease in cholesterol was a transient rise in neutral fat averaging 48 per cent. Unfortunately, all subjects but one experienced upper or lower gastrointestinal complaints, which consisted of pyrosis or dysphagia in nine, diarrhea in eight, and transient vomiting in two. Liver function studies (cephalin flocculation, thymol turbidity, alkaline phosphatase, and bilirubin) and blood counts did not change.

The compound β -diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525A), previously known as a potentiator of various drugs, including hypnotics, analgesics, spinal cord depressants, and central nervous system stimulants, has been shown by Dick *et al.* (75, 76) to have considerable hypocholesterolemic effect in dogs. Both plasma total cholesterol and aortic cholesterol were reduced. The authors state that biosynthesis of cholesterol from mevalonate in liver homogenates is inhibited by the drug, but no supporting data were given. Chronic administration of the drug to dogs resulted in a marked fatty infiltration of the liver which was reversible when the compound was withdrawn.

INTERRUPTED BIOSYNTHESIS OF CHOLESTEROL

It has been known for some time that a wide variety of insects have a nutritional requirement for sterols. In the case of the larvae of the beetle *Dermestes vulpinus*, this nutritional requirement is highly specific and is met only by cholesterol, some cholesterol esters, 7-dehydrocholesterol, or 24-dehydrocholesterol (77, 78). Preliminary studies (79) indicated that the pathway of biosynthesis is blocked in the larvae of this species between squalene and lanosterol, since a labelled material with the chromatographic behavior of squalene could be isolated from the non-saponifiable fraction of the larvae following growth on labelled acetate. A more extended study by Clark & Bloch (80, 81) has now shown that this material is not squalene, since a hexahydrochloride derivative could not be formed. Apparently cholesterol biosynthesis in this organism is blocked at a number of stages, since the cholesterol necessary for growth of the larvae cannot be replaced or spared by mevalonic acid, squalene, lanosterol, Δ^4 -4,4-dimethylcholesterol, ergosterol,

bile acids, Δ^5 -pregnenolone, or vitamin D₃. The cholesterol requirement is spared, however, by cholestanol, Δ^7 -cholestenol, 22-dehydrocholesterol, Δ^7 -ergosterol, and, to a somewhat lesser extent, by stigmasterol, 22-dihydroergosterol, and $\Delta^7,22$ -ergostadienol. Clark & Bloch conclude that the sterols that can spare, but cannot replace, cholesterol in the nutrition of *Dermestes vulpinus* serve in some structural, rather than metabolic, function.

Utilization of mevalonic acid-2-C¹⁴ has been studied by Wootton & Wright (82) in *Lumbricus terrestris* (the common earthworm). Animals were made as free of symbionts as possible by maintenance in moist cellulose powder containing "Moldex" (sodium propionate) and penicillin for six days before use. Mevalonic acid-2-C¹⁴ was injected into the alimentary tract, and after 24 hr. the worms were saponified and a non-saponifiable fraction was obtained. In one experiment, carrier squalene was added following preliminary separation of the squalene by chromatography of the fraction on alumina. The hexahydrochloride of squalene was prepared and recrystallized to a theoretical C, H, and Cl analysis. This derivative showed considerable radioactivity. In a second experiment, carrier squalene was added prior to chromatography. The hexahydrochloride of squalene was formed and recrystallized several times without significant change in radioactivity. Another radioactive product behaved like carotene, when chromatographed on alumina, but β -carotene added as a carrier was recovered without radioactivity. Similarly, coenzyme Q₁₀ added to the radioactive fraction was recovered without activity. A digitonide prepared from the non-saponifiable fraction of the injected worms was also inactive. It is concluded that sterol biosynthesis is blocked in this species at a stage between squalene and β -hydroxy sterols. It may be predicted from the data presented that the common earthworm has a dietary requirement for sterols.

FACTORS INFLUENCING THE BIOSYNTHESIS OF CHOLESTEROL IN VIVO AND IN VITRO

Adenosine triphosphate.—Wright and collaborators (3, 83) have shown that homogenates of rat liver preincubated with ribonuclease lose the capacity to convert mevalonic acid into non-saponifiable material and cholesterol. This loss of synthetic capacity is associated with the failure of ribonuclease-treated homogenates to maintain a functional level of ATP (84, 85). Inactivation of homogenates may be prevented or reversed by a Dowex-1-treated autoclaved extract of fresh liver (86, 87). The active component from liver appears to be ribonucleoprotein (88, 89). The effect is non-specific, however, and the liver extract factor may be duplicated with commercial DNA, large amounts of commercial RNA, or even by unrelated polyanions, such as heparin or polyethylene sulfonate (90). Interpretation of the data obtained with various "unnatural" polyanions is complicated by the fact that these compounds are also inhibitors of ribonuclease. Homogenates of rat liver preincubated with certain polycations, such as protamine or polylysine, also lose the capacity to convert mevalonic acid into non-saponifiable

material and cholesterol (91). Here again, as with ribonuclease, this loss of synthetic capacity is associated with the failure of protamine-treated homogenates to maintain a functional level of ATP, and the inactivation likewise may be prevented by polyanions. However, when ribonuclease is denatured by acid and heat under conditions in which enzymatic activity is largely lost without significant alteration of acid-base properties, the capacity to inactivate homogenates disappears (92). It has been suggested that liver homogenates ordinarily contain a polynucleotide, probably largely combined as a ribonucleoprotein, which is essential for the maintenance of the ATP levels that are necessary for utilization of mevalonic acid. Apparently this polynucleotide or RNA may be inactivated by ribonuclease (in association with other enzymes of a whole homogenate) simply by degradation to small fragments or inactivated by polycations by precipitation of polycation-RNA complexes. It would appear that possibilities exist for the control of cholesterol biosynthesis *in vivo* by some regulation of tissue levels of ATP. (See the following section for a possible means by which this regulation might be accomplished.)

Vanadium salts.—A number of papers in recent years have described the inhibition of cholesterol biosynthesis by vanadium salts, and the status of these studies has been summarized (93). It has been claimed that vanadium salt inhibition is obtained *in vivo* with the intact animal (94 to 97) as well as in *in vitro* experiments (98, 99). The inhibition involves reactions between mevalonic acid and cholesterol, but the exact locus had not been more specifically defined. It has now been shown by Wright *et al.* (100) that homogenates pretreated with vanadyl sulfate, in contrast to controls, are devoid of ATP. These results indicate that the site of vanadyl inhibition of cholesterol biosynthesis, at least as far as liver homogenate is concerned, is in reactions involving the maintenance of ATP levels. These *in vitro* studies with vanadyl sulfate are of further interest because preliminary results, obtained by several groups, indicate a reduction of cholesterol biosynthesis *in vivo* both in man and lower animals without evidence of toxicity following administration of vanadium salts. If this can be confirmed and if the mechanism described here is operative in the intact animal, it follows that (a) ATP levels of tissues *in vivo* may be altered by dietary means; (b) in the presence of marginal amounts of ATP, cholesterol biosynthesis is inhibited before other more essential ATP-requiring reactions are influenced; and (c) a search should be made for useful compounds that lower tissue levels of ATP, since such compounds might be expected to have utility in the control of hypercholesterolemia and related sequelae. Cholesterol biosynthesis in liver homogenates also is inhibited by other "trace elements," some of which are even more active than vanadium (101), but the mechanism of inhibition may not necessarily be the same. It is interesting to speculate on the extent to which the increase in the incidence of atherosclerosis and other conditions accompanied by elevated blood levels of cholesterol might be correlated with the consumption of diets increasingly deficient in trace elements.

Nicotinic acid.—Altschul *et al.* (102), in 1955, reported that a reduction of serum cholesterol concentration occurs in normal and hypercholesterolemic individuals following the ingestion of relatively large amounts of nicotinic acid (1 to 4 gm in 24 hr). Since that time, literally scores of papers, including a number of reviews (103 to 106), have appeared dealing with the phenomenon. Although the mechanism of the hypocholesterolemic action of nicotinic acid is by no means settled, nicotinic acid may have an inhibitory effect on cholesterol biosynthesis.

Perry (107) has reported that incubation of rat liver slices with added nicotinic acid (1 mg. per ml.) is associated with a considerable reduction in the incorporation of acetate into cholesterol. This decrease in the conversion of acetate to cholesterol was accompanied by an increase in the conversion of labelled acetate to labelled carbon dioxide. No effect of nicotinic acid was observed on the incorporation of acetate into fatty acids. Nicotinamide (1 mg. per ml.) was without effect on the biosynthesis of cholesterol or the production of carbon dioxide from acetate, but nicotinamide depressed incorporation of acetate into fatty acids. Similarly, Schade & Saltman (108) have shown with rabbits on a stock ration and rabbits on a stock ration plus 2 per cent cholesterol that the further addition of nicotinic acid at a level of 0.5 per cent to either diet is associated with a reduction in the biosynthesis of cholesterol from acetate by liver slices. Schade & Saltman suggest that the feeding of nicotinic acid is associated with a decreased biosynthesis of cholesterol because coenzyme A is diverted from its role in the activation of acetate to the activation of nicotinic acid essential for detoxification and excretion as nicotinuric acid.

Preliminary results by Parsons (103) on acetate incorporation in humans show considerably less conversion into serum cholesterol (free and esterified) and erythrocyte cholesterol during nicotinic acid administration. On the other hand, Hardy *et al.* (109) observed that liver slices from rats or chicks fed nicotinic acid showed an increased incorporation of acetate into sterols and a decreased incorporation into fatty acids. Similar results were obtained when nicotinic acid was added to liver slices *in vitro*. This effect was attributed to a shift of the labelled substrate from fatty acid to sterol biosynthesis. Duncan & Best (110) also reported no effect of nicotinic acid feeding on the incorporation of label from acetate-1-C¹⁴ into serum or liver cholesterol in rats that had been fed a stock diet containing 1 per cent added nicotinic acid for eight or 42 days *ad libitum* or for 42 days where the control diet was paired with the nicotinic acid-containing diet. The precursor (25 to 30 mg) was administered intraperitoneally 2 hr before sacrificing the animals.

CAROTENOIDS

As a result of studies involving a variety of organisms, including *Phycomyces blakesleeana* (111), *Mucor hiemalis* (112), carrot root (113), and tomato fruit (114), it has been known for some time that acetate serves as a precursor of carotenes. For example, Grob & Büttler (112) found that carotene

derived from *Mucor hiemalis* following growth on acetate-1-C¹⁴ or acetate-2-C¹⁴ was radioactive and the pattern of labelling was the same as that encountered by Cornforth & Popjak (115) in radioactive squalene derived from labelled acetate. Similarly, carotene obtained from *Phycomyces blakesleeanus* following growth on acetate-2-C¹⁴ was found by Braithwaite & Goodwin (111) to be radioactive, and the labelling was that expected from the studies of Cornforth & Popjak (115).

Again, despite the atypical labelling reported by Zabin (114) in lycopene synthesized by intact tomatoes from labelled acetate, Braithwaite & Goodwin (116) have now shown that the anomalous results were the result of the use of an inadequate method of degradation of the labelled pigment, and new results with improved methods show that acetate incorporation into lycopene by tomato slices follows that labelling pattern encountered in all other terpenoid compounds examined. With the advent of mevalonic acid, biosynthesis of carotenes could be studied in greater detail.

With respect to the labelling encountered in carotene derived from labelled mevalonic acid, the balance of evidence indicates that mevalonic acid is incorporated without randomization according to the isoprene rule. Such studies have been carried out both with *Phycomyces blakesleeanus* (113, 117) and *Mucor hiemalis* (118). Except where the factor of permeability is involved, mevalonic acid is severalfold more active than acetate as a precursor of carotenes. For more extended discussions of the earlier work on carotene biosynthesis, the reviews of Grob (119) and Goodwin (120, 121) may be recommended.

Yokoyama *et al.* (122) have recently described the preparation of a cell-free extract of *Phycomyces blakesleeanus* capable of synthesizing labelled carotene from acetate-2-C¹⁴, β -hydroxy- β -methyl glutarate-3-C¹⁴, and mevalonate-2-C¹⁴. They also obtained evidence for the back reaction of mevalonate to β -hydroxy- β -methyl glutarate.

Anderson *et al.* (123) have shown that, while mevalonic acid-2-C¹⁴ is incorporated into carotenes by the ripening tomato fruit, great care must be exercised in characterizing the products, since non-saponifiable companions with higher activity contaminate the carotenes and are not necessarily removed by chromatography or crystallization. Careful studies of Anderson *et al.* (124) have shown that mevalonic acid is incorporated into phytoene, phytofluene, ζ -carotene, neurosporene, lycopene, β -carotene, and γ -carotene in ripening tomatoes. In addition, mevalonic acid is incorporated into several unknown, non-carotene, non-saponifiable compounds that do not have the properties of intermediates in the biosynthesis of squalene and do not appear to be intermediates in the biosynthesis of carotenes.

A number of compounds have been compared by Krzeminski & Quackenbush (125) as precursors of carotenes in growing cultures of *Neurospora crassa*. Mevalonic acid was superior to other possible precursors studied, including acetate, pyruvate, glycerol, alanine, phenylalanine, and glucose. Their claim that the N,N'-dibenzylethylenediamine salt of mevalonic acid is

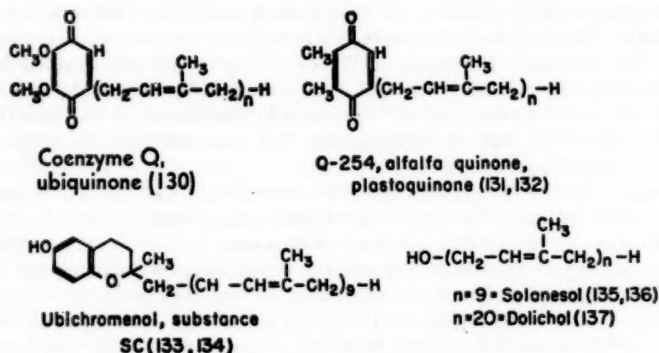


FIG. 7. Some members of coenzyme Q family.

superior to the sodium salt in experiments involving the use of the labelled precursors dissolved in complex media is difficult to understand.

Complete papers of Braithwaite & Goodwin have now appeared describing the incorporation of acetate and mevalonate into carotenes of *Phycomyces blakesleeanus* (117) and cell-free carrot-root preparations (126). It would seem that the stage is now set for a study of carotene biosynthesis by purified enzyme systems.

COENZYME Q FAMILY

The coenzyme Q family has been enlarged with the isolation of a number of new compounds. All possess a polyisoprenoid moiety. It has not been completely resolved whether or not ubiquinone is an artifact of isolation (127, 128, 129). The structure of some of the compounds (130 to 137) are summarized in Figure 7.

Gloor & Wiss (138) have administered mevalonic acid-2- C^{14} to normal rats and rats in various stages of vitamin-A deficiency and have isolated coenzyme Q_{10} from livers with the aid of carrier compound. Mevalonic acid is definitely incorporated into coenzyme Q_{10} . Similarly, Dialameh & Olson (139) have shown incorporation of label from acetate-1- C^{14} into coenzyme Q_{10} following intraperitoneal administration of the precursor to normal rats and isolation of the product with carrier. The incorporation increases with the severity of vitamin-A deficiency. Presumptive evidence was obtained indicating that incorporation of mevalonic acid into squalene but not cholesterol is increased in the deficiency. Tritiated 2,3-dimethoxy-5-methyl-1,4-benzoquinone was prepared and administered to rats. No incorporation into coenzyme Q was observed, thus confirming the statement of Martius & Esser (140) that the parent quinone is not a precursor of coenzyme Q. Subsequent study by Gloor & Wiss (141) has confirmed the fact that incorporation of

mevalonic acid into squalene is increased in vitamin-A deficiency. On the other hand, incorporation of mevalonic acid into cholesterol appears now to be decreased in the deficiency. Incorporation of mevalonic acid into coenzyme Q, squalene, and cholesterol approached normal rates when mevalonic acid was administered to rats 20 hours after they received a single dose of vitamin A following 3½ weeks of depletion.

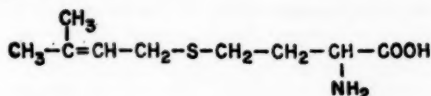
Improved methods for the separation of coenzymes Q₉ and Q₁₀ (142) have been used by Olson and co-workers (143, 144) in studies on the incorporation of acetate-1-C¹⁴ into these compounds by the intact rat. Incorporation of acetate into coenzyme Q₉ is about 30 times that into Q₁₀. Olson & Dialameh conclude that coenzyme Q₉ is the endogenous form of the coenzyme in normal rat liver and that the Q₁₀ present is derived largely from microfloral or dietary sources. Degradative studies have indicated that the incorporation is exclusively into the side chain of the quinone (144). Similarly, studies with mevalonic acid-C¹⁴ have been carried out by Gloor & Wiss (135), who conclude that coenzyme Q₉ is the predominant form of quinone in rat liver. Others have reached the same conclusions (145). In addition, Gloor & Wiss (135) have administered mevalonic acid-C¹⁴ to rats and subsequently isolated solanesol (the isoprenoid side chain of coenzyme Q₉) from the livers with the aid of carrier compound. The isolated solanesol proved to be devoid of activity. This finding led to the conclusion that solanesol, as encountered in animal tissues, is derived from dietary sources rather than from biosynthesis within the animal.

Rudney & Sugimura (146) have isolated coenzyme Q₈ (Ubiquinone 30), presumably labelled in the methoxyl groups, from yeast grown on formate-C¹⁴ and have shown that this compound is not converted to coenzyme Q₉ when administered to rats. Apparently there is little, if any, lengthening of the isoprenoid moiety once it is attached to the benzoquinone.

MISCELLANEOUS COMPOUNDS

With the advent of mevalonic acid, renewed interest has been shown in the biosynthesis of compounds with structures that indicate an origin from "biologically-active" isoprene. A number of papers have appeared demonstrating the incorporation of mevalonic acid according to the "isoprene rule" into rubber (147, 148), mycelianamide and mycophenolic acid (149 to 152), gibberellic acid (152), rosenonolactone (152), felinine (153), tricothecin (152), pinene (154), citronellal and cineole (155), and soyasapogenol (156).

In a review of the biosynthesis of some of the above compounds by fungi, Birch (see 152) has postulated the existence of a mechanism of "transisopentenylation" analogous to transmethylation, involving the following compound as "isopentenyl donor."



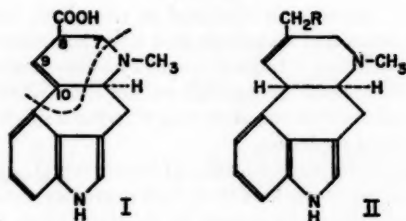


FIG. 8. Ergot alkaloids originating in part from mevalonic acid.

Additional study is required to determine whether or not the recent electronic interpretations for the condensation of dimethylallyl pyrophosphate with isopentenyl pyrophosphate (31, 33) require elaboration to the extent of involving such an intermediate. More recently, a number of additional compounds have been shown by labelling experiments to originate from mevalonic acid.

Digitoxigenin.—Following the injection of mevalonic acid-2- C^{14} into a healthy plant of *Digitalis lanata* by Ramstad & Beal (157), the lanatoside A fraction was isolated by paper chromatography and subjected to Mannich hydrolysis. Radioactivity encountered in the lanatoside A fraction was found exclusively in the genin portion of digitoxigenin rather than in the sugar portion.

Ergot alkaloids.—Tryptophan has been known to be a precursor of the ergot alkaloids in Figure 8, lysergic acid (I), agroclavine (II; R=H), and elymoclavine (II; R=OH), but the source of the five "non-tryptophan" carbons was not determined. Birch *et al.* (158) have now shown that in all probability these carbons arise from mevalonic acid.

In the study by Birch *et al.*, *Claviceps purpurea* was grown on media containing mevalonic lactone 2- C^{14} , acetate-1- C^{14} , and acetate-2- C^{14} , and the resulting agroclavine and elymoclavine were isolated and degraded. Radioactivity was encountered in position 17 from mevalonic acid-2- C^{14} , in position 8 from acetate-1- C^{14} , and in position 17 from acetate-2- C^{14} ; this is the distribution of radioactivity expected from the isoprene rule (159).

REVIEWS

A number of excellent reviews of varying length concerning the biosynthesis of the terpenoids and sterols have recently appeared. Those of Cornforth (47), Popjak & Cornforth (28), Todd (1), Kritchevsky *et al.* (160) are especially recommended. The best single reference is *Biosynthesis of Terpenes and Sterols* (Little, Brown & Company, Boston, Massachusetts, 1959) to which frequent attention has been called in this review.

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PORPHYRINS AND HEMOPROTEINS^{1,2,3}

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Heme pigments and porphyrins were last reviewed in these volumes by Rimington (1) in 1957. In the intervening period, in addition to major advances in the elucidation of the enzymic steps in the biosynthesis of heme, a large body of knowledge on hemoproteins has accumulated (2), but it is necessary to restrict the present article largely to structural and biosynthetic aspects. Functional phenomena will be considered only insofar as they bear on the relations of structure to activity. The principal areas not covered are: the co-ordination chemistry of heme compounds and the ligand field theory [see reviews by Griffith & Orgel (3), Orgel (4, 5) and Williams (6 to 8)], which have led to remarkable correlations of spectra, magnetic properties, and oxidation-reduction potentials in model systems and their application by analogy to the structure of hemoproteins (7); higher oxidation states of iron as a theory of heme catalysis [see review by George & Griffith (9)]; the chemistry and biosynthesis of chlorophyll and of the bile pigments. Cytochromes- $a+a_3$, $-b$, $-b_2$, $-b_5$, and $-c_1$, as well as catalases and peroxidases, have been reviewed by Morrison & Stotz (10).

THE BIOSYNTHESIS OF PORPHYRINS

The major stages in the biosynthesis of porphyrin were established after Shemin & Rittenberg (11) showed that N^{14} -glycine labelled the heme of hemoglobin. The exact mechanism of a number of particular steps is still in doubt. Since the 1955 CIBA Foundation Symposium (12), the field has been thoroughly reviewed by Rimington in 1957 (1) and 1959 (13), and specific aspects have been reviewed by other workers (14 to 17).

¹ The survey of most of the literature pertaining to this review was concluded in October, 1960.

² The following abbreviations are used: CoA for coenzyme A; Hb for hemoglobin [the abbreviations for the human hemoglobins are those given in *Blood*, 8, 386 (1953); HbA is the main normal adult Hb; HbA₂ is the minor component of normal adult human blood described by Kunkel & Wallenius (192); HbF is fetal Hb; HbS is sickle-cell Hb, etc.]; Mb for myoglobin; —SH for sulfhydryl.

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Formation of δ -aminolevulinic acid.—The demonstration that δ -aminolevulinic acid is an intermediate in porphyrin synthesis both in animal tissues (18 to 23) and in microorganisms (24 to 26) was mainly based on isotope-dilution experiments and on the observation that δ -aminolevulinic acid is a more active precursor of heme, porphobilinogen, and porphyrins than glycine in a number of *in vitro* systems (27 to 29). It was postulated that succinyl-CoA is involved in porphyrin synthesis (30) and couples with glycine to form α -amino- β -ketoadipic acid which then spontaneously or enzymically decarboxylates to give δ -aminolevulinic acid (18). The evidence for the involvement of succinyl-CoA was that the labelling pattern of heme synthesized from C^{14} -acetate indicated that the acetate was first converted to a four carbon unsymmetrical compound through the citric acid cycle (31). Further evidence was obtained by showing that heme synthesis from glycine and succinate was depressed in hemolysates of pantothenate-deficient ducklings, whereas it was unaffected from δ -aminolevulinic acid (32). A study of porphyrin synthesis in *Tetrahymena vorax* (25) gave similar results.

The citric acid cycle has been related to porphyrin formation by the use of succinate, α -ketoglutarate, and citrate labelled in various positions (30, 33). Relationships of these and other factors to protoporphyrin synthesis in chicken red cells and hemolysates have also been studied (34).

The net synthesis of δ -aminolevulinic acid occurs in particle-free extracts of *Rhodospseudomonas spheroides* (35 to 37), in particulate preparations from erythrocytes of phenylhydrazine-treated hens (38), and in normal chickens (39). Further purification of these preparations have made possible the direct demonstration that succinyl-CoA is indeed an intermediate in the synthesis of δ -aminolevulinic acid and that pyridoxal phosphate is required, presumably to "activate" the glycine in the form of a pyridoxal phosphate derivative (39 to 41). The participation of pyridoxal phosphate in this reaction was first indicated by Schulman & Richert (32), who obtained with pyridoxine-deficient ducklings results similar to those quoted above for pantothenic acid deficiency. Ferrous ions are also apparently required for the reaction (42). The role of iron in the biosynthesis of heme has been studied in blood from iron-deficient ducklings (43). It was shown that iron is implicated in two major steps: the synthesis of δ -aminolevulinic acid and of heme from protoporphyrin. Whether α -amino- β -ketoadipic acid is a transient intermediate in the formation of δ -aminolevulinic acid (19) is not settled (40). The enzyme may carry an essential —SH group (40). Extracts of *R. spheroides* appear to contain an inhibitor of the reaction (41), and inhibitions by a number of normal tissue metabolites have also been observed (39).

Formation of porphobilinogen.—The substituted monopyrrole porphobilinogen is an intermediate in porphyrin synthesis in numerous different systems (18, 23; 43 to 54). The condensation of two molecules of δ -aminolevulinic acid with the elimination of two molecules of water to form porphobilinogen has been extensively studied with purified preparations of the aminolevulinic acid dehydrase, the enzyme responsible for the condensation (27,

28, 29, 50, 55, 56, 57). This enzyme is widely distributed in animals, plants, and microorganisms (28); it is readily water-soluble and is a typical sulfhydryl enzyme since it is inhibited by sulfhydryl reagents and requires activation with reducing agents, such as reduced glutathione (28, 50). It is inhibited by ethylenediaminetetraacetic acid (28, 50) and δ -chloro- or δ -oximino-levulinic acid (58). The two steps of the condensation reaction are, respectively, an aldol type condensation and a Schiff base linkage. Whether only one or both steps are enzymically catalyzed is uncertain. There is little doubt, however, that a single protein is sufficient to carry out the complete reaction (28) and that it specifically binds both molecules of δ -aminolevulinic acid (50). The aminolevulinic acid dehydrase of liver has been found to contain copper (59, 60) and magnesium (61) and to be decreased in copper-deficient rats (59, 60). Removal of the copper did not affect enzyme activity (60). The enzyme does not appear to be related to copper-deficiency anemia (60) since copper-deficient animals have, in bone marrow, normal dehydrase activity for δ -aminolevulinic acid and a normal rate of heme synthesis *in vitro* (61). The administration of 3-amino-1,2,4-triazole has also been reported to decrease in rats the aminolevulinic dehydrase activity of liver (62). An increase in aminolevulinic acid dehydrase activity was found in *R. spheroides* during adaptation to the synthesis of bacteriochlorophyll (63).

Formation of uroporphyrinogen.—When the enzymic condensation of several molecules of porphobilinogen is carried out under aerobic conditions, the product is mainly uroporphyrin. This is not, however, the immediate product of the reaction but rather the result of a spontaneous or an enzymic oxidation of the primary product, uroporphyrinogen. In this oxidation reaction, six H atoms are removed, the four methane bridges are transformed to methene bridges, and the fluorescent, flat porphyrin molecule containing a system of conjugated double bonds is formed. Uroporphyrin cannot be utilized as substrate for the subsequent biosynthetic steps (19, 23, 47, 51, 64, 65), whereas uroporphyrinogen is readily transformed enzymically into heme or the preceding intermediates (51, 52, 65, 66, 67) and will accumulate, with partially purified enzyme preparations under suitable anaerobic conditions (50, 51, 54). Moreover, Bogorad (51) has noticed the presence of a heat-labile substance in his preparation, presumably an enzyme, that appears to catalyze the oxidation of uroporphyrinogen to uroporphyrin, and he has also obtained spectroscopic evidence for the appearance of a transient partial oxidation product that has an absorption maximum at 500 m μ [see Mauzerall & Granick (65) for the probable structure of this intermediate]. It should, however, be noted that porphyrinogens can readily auto-oxidize to porphyrins in the absence of any enzyme.

Except in pathological conditions in which mixtures of porphyrins of the I and III series are produced, as shown, for example, in recent studies of porphyrins isolated from cases of acute porphyria and porphyria cutanea tarda (68 to 70), only porphyrins of the III series are found in animals in more than trace amounts. The major difficulty in understanding the conden-

sation of porphobilinogen to uroporphyrinogen is to visualize how the unsymmetrical type III can be formed from four units of the monopyrrole porphobilinogen when a simple linear polymerization followed by ring closure would yield the symmetrical type I. Using A as a symbol of the acetyl side chain and P for the propionyl side chain, the porphyrins of the I series can be described as (AP.AP.AP.AP), whereas those of the III series would be (AP.AP.AP.PA); the substituents of the porphyrin rings A to D are written from left to right.

Numerous hypotheses, based largely on chemical analogies and the study of the non-enzymic condensation of porphobilinogen to porphyrins, have been advanced in explanation of the mechanism of the condensation reaction:

(a) Hypotheses based on the intermediate formation of branched pyrrolymethane structures: Shemin and co-workers (19) proposed an intermediate radial tripyrrylmethane structure, and a mechanism that involved the loss of one pyrrole unit as opsopyrroledicarboxylic acid and the accumulation of one mole of formaldehyde for every mole of cyclic tetrapyrrole of the III series formed. Bogorad & Granick (46, 71) considered a linear tripyrrylmethane which is oxidized to the corresponding tripyrrylmethene and then condensed with a further porphobilinogen unit [Corwin-Andrews reaction (72, 73)] to form a radial tetramer or "T" structure. This compound can then cleave and recondense to give a partially reduced type III structure. Wittenberg (74) proposed that two molecules of a linear tetrapyrrolylmethane condense head to tail by a Corwin-Andrews type reaction to form a cyclic octapyrrylmethane with six pyrroles in the ring and two outside it, thus giving two radial tripyrrylmethane structures in the same molecule. On rearranging this cyclic compound, two molecules of a type III structure are obtained; whereas, if the original linear tetramer cyclizes before condensing to the octapyrrole, type I molecules are obtained. As pointed out by Rimington (75), the Corwin-Andrews mechanism leading to the formation of a radial polypyrrolylmethane requires the methene and not the methane derivatives. Hence, all these hypotheses necessarily involve an oxidation step for the formation of methane structures from methane compounds.

(b) Hypotheses also involving oxidized derivatives other than polypyrrolymethenes: Shlyk (76) proposed the participation of porphobilinogen aldehyde in a mechanism involving either the loss of one pyrrole unit or the formation of an intermediate dipyrrolylmethene.

(c) Hypotheses involving exchange or condensation reactions of linear polymers: Bogorad (77) proposed an exchange between a porphobilinogen unit and a dipyrrolic segment of a linear tripyrrole, followed by the condensation of the two dissimilar dipyrroles to give a type III structure. Jackson & MacDonald (78) considered the condensation of opsopyrroledicarboxylic acid with one end of a linear tetrapyrrolylmethane to form a pentamer from which opsopyrroledicarboxylic acid could be cleaved from the other end. In this way, a tetramer is formed that can cyclize to a type III structure, and

opropyrroledicarboxylic acid is regenerated and acts essentially as a coenzyme. A similar mechanism in which opopyrroledicarboxylic acid was cleaved from a linear tetramer and then condensed back after rotation of the molecule onto the residual trimer was proposed by Godnev & Rotfarb (79).

(d) Hypotheses invoking the intramolecular migration of side chains: Cookson & Rimington (80) proposed a mechanism based on their study of the acid-catalyzed condensation of porphobilinogen to porphyrin, which includes the labilization or displacement of side chains and requires the formation and utilization of formaldehyde. A proposal of Treibs & Ott (81) similarly involves formaldehyde. Utilizing the same chemical approach as Cookson & Rimington (80), Robinson (82) proposed that the migration of the $-\text{CH}_2\text{NH}_2$ side chain of porphobilinogen leads eventually only to a series III compound. Similarly, Bullock *et al.* (83) proposed the migration of a methylene group following an initial carbonium ion attack on the substituted alpha-position of the second porphobilinogen unit. In this mechanism, the first interpyrrole bridge formed would be the δ -methene bridge between rings D and A of the type III structure. If the initial carbonium ion attack is on the unsubstituted position of the second porphobilinogen molecule, uroporphyrinogen I would be made. Lockwood & Benson (54), however, point out that, even if the initial carbonium ion attack is always directed against the substituted alpha-position of porphobilinogen, if an immediate ring closure occurs when a tetrapyrrole is formed, one would obtain uroporphyrinogen III, whereas, if longer polypyrroles are made, still attached to the enzyme, ring closure could give a single molecule of type III together with an indefinite number of type I molecules. Mauzerall (84) has recently studied the isomerization of uroporphyrinogen and the non-enzymic condensation of porphobilinogen in acid, neutral, and alkaline solutions. The mechanism is essentially that given by Cookson & Rimington (80), although the experimental results do not strictly exclude the intramolecular migration theories of Robinson (82) and of Bullock *et al.* (83). Chemical studies on the synthesis of various porphyrin derivatives have been reported (85 to 88). The total synthesis of chlorophyll has also been accomplished (89).

None of the theories has been proved correct, but many can be ruled out or appear improbable. The experimental evidence pertaining to the enzymic condensation of porphobilinogen to uroporphyrinogens can be summarized as follows:

(a) Most crude enzyme preparations form preferentially porphyrins of the III series (46, 90, 91). However, on preheating the enzymically active extracts, the ability to form intermediates of the III series is lost, and only those of the I series are made (46, 91). Similarly, in a normal rat liver homogenate, the conversion of porphobilinogen to uroporphyrinogen III during the early stages of the incubation gives way later to the synthesis of type I (92). Granick & Mauzerall (50) separated from erythrocytes an enzyme fraction that catalyzed the formation of type III from porphobilinogen, and they also found that after preheating only type I was formed. Bogorad (47, 48, 51)

has obtained an enzyme preparation from spinach named "porphobilinogen-deaminase," which forms only type I and stoichiometric amounts of ammonia; another partially purified preparation from wheat germ, called "uroporphyrinogen isomerase," has no effect on porphobilinogen, but, in the obligatory presence of both porphobilinogen and the deaminase, it catalyzes the formation of uroporphyrinogen III. Type I, however, is not an intermediate in the biosynthesis of type III (93). Preparations of the deaminase, which had essentially the properties described by Bogorad, were obtained by Hoare & Heath (67) from *R. spheroides*. On the other hand, Lockwood & Rimington (49) and Lockwood & Benson (54) found no evidence for the presence of two enzymes in preparations from avian erythrocytes. Their preparation, "porphobilinogenase," formed uroporphyrinogen III from porphobilinogen; after heating or in the presence of cyanide or azide only type I was made. The recent observations of Falk & Dresel (94) are pertinent in regard to the question of the implication of one or two enzymes. These authors found that with porphobilinogen as substrate, unhemolyzed erythrocytes from chicken, normal rabbit, and normal human blood preferentially formed type I. On the other hand, with δ -aminolevulinic acid, a substance which is permeable to red cells, as opposed to porphobilinogen which is apparently not, these preparations carried the biosynthesis to the III series compounds. This suggests a difference in the cellular distribution of those enzymes that can form uroporphyrinogen I and those that lead to the III series porphyrins. The separation of enzymes forming series I compounds from factors required to carry the synthesis to the type III derivatives in chicken erythrocyte hemolysates has been reported (95).

(b) Only four molecules of porphobilinogen are required to form one of uroporphyrinogen. There is no loss of one or more units either in the enzymic (23, 50, 51, 54) or the acid-catalyzed condensation of porphobilinogen to porphyrins (80). These findings invalidate the hypothesis of Shemin *et al.* (19) [see (a) above].

(c) No oxidation occurs before the final cyclization to porphyrinogen, the enzyme or enzymes forming types I or III being fully active under anaerobic conditions (50, 51, 54). Thus, the hypotheses that require an early oxidation step, all three given under (a) on page 552 and that listed under (b) on page 552, do not fit the experimental findings. Nevertheless, one might consider that the two H atoms removed in order to permit the condensation to a branched polypyrrolylmethane structure are added back in the subsequent cleavage of the radial structure, thus involving no net oxidation. However, such a situation is unlikely.

(d) Free formaldehyde is neither a stoichiometric by-product nor a reactant in the enzymic synthesis of porphyrinogens (54, 96), even though it is formed and utilized in the acid-catalyzed condensation of porphobilinogen to porphyrins (19, 54, 96). These findings eliminate the hypothesis of Shemin *et al.* (19) and, to a large extent, those of Cookson & Rimington (80) and of Treibs & Ott (81) [see (d) on page 553] even though they do not entirely ex-

clude the possibility of a migration of enzyme-bound formaldehyde from one pyrrole ring to another if one assumes that the enzyme-bound formaldehyde cannot exchange with free formaldehyde.

(e) Opsopyrroledicarboxylic acid is not incorporated into heme by lysates of fowl erythrocytes (97), and thus the hypothesis of Jackson & MacDonald (78) and of Godnev & Rotfarb (79) [see (c) above] can be excluded.

(f) The tripyrrolic pigment prodigiosin is not an intermediate in the biosynthesis of porphyrins (99).

In conclusion, the only hypotheses to which no objection can be raised are those that have not yet been tested experimentally. Such tests would require the chemical synthesis of various postulated intermediates to be used as substrates. A first step in this direction has been taken by Hoare & Heath (98), who find that a dipyrrolylmethane (AP.PA) containing aminomethyl α, α' substituents is condensed by crude enzyme preparations from *R. sphaeroides* into porphyrins of the unnatural II series, whereas dimers of various configurations that have no α substituents are not utilized at all.

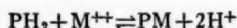
One of the unresolved problems in the enzymic condensation of porphobilinogen to uroporphyrinogen is the appearance in some cases of a substance named "pseudoporphyrin" in amount equivalent to that of the uroporphyrin formed (17, 90, 100). Pseudoporphyrin is not one of the uroporphyrin isomers but belongs to the III series since on decarboxylation it yields only coproporphyrin III (90). Falk & Dresel (94) have recently suggested that pseudoporphyrin may be an oxidation product of a cofactor-complex of uroporphyrinogen III. The requirement for a cofactor in the uroporphyrinogen III to coproporphyrinogen III transformation is discussed in the next section. Pseudoporphyrin has apparently not been detected with purified systems from red blood cells (50) or wheat germ (51) or crude systems from *Chlorella* (51) and *R. sphaeroides* (53).

Formation of coproporphyrinogen III.—The transformation of uroporphyrinogens I or III to the corresponding coproporphyrinogens requires the decarboxylation of the four acetyl side chains of the former. As noted above, only the uroporphyrinogens and not the uroporphyrins will undergo enzymic decarboxylation (51, 52, 65, 66, 67). The enzyme functions anaerobically (51, 65), can decarboxylate all of the four possible isomers of uroporphyrinogen, and in the reaction liberates intermediate products having seven, six, and five carboxyl groups (51, 65, 67). Porphobilinogen, δ -aminolevulinic acid, uroporphyrin, and coproporphyrinogen are not affected by the purified enzyme (65). It is a typical sulfhydryl enzyme which is readily inactivated in the absence of reduced glutathione (65) or dimercaptopropanol (67) and by heavy metals, iodoacetamide, and *p*-chloromercuribenzoate. A heat-stable, low molecular weight cofactor may be important in this region of the biosynthetic pathway. Falk *et al.* (17) have shown that a brei of ineffective root nodules of soybean does not form uroporphyrin, coproporphyrin, and protoporphyrin except in the presence of boiled yeast juice or of a boiled extract from effective root nodules. Similarly, Hoare & Heath (53, 67) have observed,

with preparations from *R. spheroides*, that the formation of coproporphyrinogen III from porphobilinogen was stimulated by a heat-stable, ultrafiltrable factor from the microorganism, chicken blood, bakers' yeast or pig liver.

Formation of protoporphyrin.—The conversion of coproporphyrinogen III to protoporphyrin requires the transformation of the two propionyl side chains on rings A and B into vinyl groups, a process involving decarboxylation as well as oxidation, and the oxidation of the porphyrinogen to the porphyrin. Less is known of these steps than about any others in the biosynthesis of heme. The enzymes that catalyze these reactions appear to be attached to cellular particles. Dresel (101) and Dresel & Falk (23) observed that particle-free supernatants from avian hemolysates carried the biosynthesis from porphobilinogen no further than coproporphyrinogen, whereas the complete hemolysate readily formed protoporphyrin. The particle-bound enzyme system could also be supplied in the form of rat liver mitochondria, and oxygen was necessary for the reaction. Essentially similar experiments by Booi & Rimington (91) showed that the mitochondria need not be intact (13). Unhemolyzed avian erythrocytes and mammalian reticulocytes which contain mitochondrial processes can form protoporphyrin from δ -aminolevulinic acid, whereas non-nucleated mammalian erythrocytes can only form coproporphyrinogen (94). Granick & Mauzerall (102) reported that particulate preparations from red cells and *Euglena* transform coproporphyrinogen III, but not I, into protoporphyrin; the oxidation to the porphyrin probably occurred from protoporphyrinogen, which is more readily auto-oxidizable than coproporphyrinogen (71); an intermediate tricarboxylic substance was detected; oxygen was necessary; cyanide, iodoacetamide, dinitrophenol, and ethylenediaminetetraacetic acid did not inhibit the reaction; CoA and ATP did not stimulate it (102).

Incorporation of iron.—Ferrous iron readily reacts with protoporphyrin to yield heme in acetic acid solution. However, once formed, porphyrin-metal complexes are unusually stable; the metal does not exchange measurably with similar ions in solution (103, 104). Some recent experiments have clarified the reason for this situation. Dempsey *et al.* (105), studied the kinetics of the reaction of Zn^{++} with monomolecular dispersions of various fully esterified porphyrins in detergent solutions, and they concluded that there was a bimolecular displacement reaction involving the metal ion (M^{++}) and the neutral porphyrin species (PH_2)



and not a dissociation-type reaction. Moreover, Fleischer & Wang (106) have shown that the metal first combines with the porphyrin to give a structure in which the metal ion is fixed on top of the flat porphyrin molecule; this intermediate then decomposes to the normal metalloporphyrin and $2H^+$.

It has often been assumed that in the biosynthesis of heme this reaction proceeded non-enzymically under normal *in vivo* conditions of pH and tem-

perature; indeed, when protoporphyrin is monomolecularly dispersed in aqueous solutions containing surface-active agents, the co-ordination with Fe^{++} to form heme can readily be observed (107). A suggestion that porphyrinogens (108) might be acceptors for iron to form the corresponding metalloporphyrins has not been accepted (13, 65, 107). There is little doubt that protoporphyrin is the direct precursor of heme and that the insertion of iron is enzymically catalyzed (109 to 121). The enzyme has been called "heme synthetase," (122) and crude active preparations have been obtained from rat liver (114, 115, 120), chicken (118, 123, 124), or duck (111, 117) erythrocytes. In all cases, the enzyme is attached to particulate cellular fractions and can be extracted either with surface active agents (111, 115, 121, 125) or mechanical dispersion (123, 124). Particularly after dialysis or ageing, the enzyme is activated by reducing agents such as cysteine or reduced glutathione (111, 115, 118, 119, 120), and by anaerobiosis (111, 125, 126).

Schwartz *et al.* (123) have found that the addition of an inactive supernatant obtained from a chicken erythrocyte hemolysate to their enzyme preparation resulted in a several-fold increase in activity. This supernatant could be replaced by globin, and the product was hemoglobin (Hb) (124). Minakami *et al.* (127) similarly showed that if a supernatant from duck erythrocytes was added to the enzyme the main product was duck Hb, whereas if the supernatant was from horse red blood cells the product was horse Hb. Minakami *et al.* (128), however, also reported that, if their cholate-extracted enzyme preparation was freed from globin by column chromatography, the main product of the reaction was not hemoglobin but may have been free heme or heme bound to a protein other than globin. Thus, the question as to whether the presence of globin is essential for the formation of heme remains unanswered. In the experiments of Schwartz *et al.* (124), globin may have formed a globin-protoporphyrin complex (129), which would represent a specific reactant in the enzyme-catalyzed incorporation of iron, as had been suggested by the experiments of Ericksen (130) on the relative rates of formation of protoporphyrin and heme by rabbit reticulocytes. On the other hand, the globin may have served simply to solubilize the protoporphyrin, thus making it available to the enzyme, an effect that has also been observed in the non-enzymic formation of heme (107).

HEMOGLOBINS AND MYOGLOBINS

The study of the hemoglobins and related substances has led to some of the most interesting advances during the last decade. There is a multiplicity of both abnormal and normal human and animal hemoglobins, and this was followed in many cases by the discovery of specific differences in amino acid sequences. X-ray crystallography can lead to a precise picture of the three-dimensional structure of fairly complex proteins and even to the identification of the primary structure or sequence of the amino acid residues in the peptide chains of a protein. A simple method has been developed, the so-called "finger-printing" technique, for identifying differences in the amino

acid sequences of related proteins, and another for studying the identity and modalities of recombination of subunits of different hemoglobins, the "hybridization" technique. The following account will be confined to some of the recent developments and to areas only partially covered in Manwell's extensive review (131), which is written from the point of view of comparative physiology.

Physicochemical studies.—Tanford (132) suggested that the increase in acid binding on reversible denaturation of horse Hb is not caused by the "unmasking" of 36 groups per molecule (133) but by a large decrease in the electrostatic interaction factor w which accompanies the expansion or dissociation of the protein. However, Beychok & Steinhardt (134) have recently shown that changes in w do not account entirely for the observed effects. Different products are formed at 0° and 25°, the formation of both being accompanied by the unmasking of 22 groups (135). The regeneration reaction is complicated by the appearance of a transient intermediate (136). Further evidence of the complexity of the reversible acid denaturation of ferrihemoglobin is provided by a study of the calorimetrics of the reaction by Forrest & Sturtevant (137), who observed a heat evolution of 76 kcal/mole at 15° as compared to a heat absorption of 10 kcal/mole at 25°. These changes in heat capacity indicate the involvement of carboxylate ions, which together with imidazole groups (135) probably constitute the 22 groups unmasked. The number, reactivity, and function of —SH groups in Hb has been the object of numerous investigations and has been critically reviewed by Cecil & McPhee (138). There is no doubt today that HbA contains only six —SH groups (139 to 141), two of the eight silver atoms bound by the denatured protein (142, 143) being attached to functions other than —SH groups. A recent confirmation of this conclusion has come from amino acid analyses of the separated α - and β -chains which contain one and two cysteine residues respectively, making a total of six per molecule (144).

Murayama (145), continuing a series of amperometric studies of silver and mercury binding, has demonstrated that, as for other hemoglobins, in HbA there is an interaction between the binding sites for heavy metals. The "mercapto-mercapto" interaction constant m is defined by an equation similar to the well-known Hill equation (146) used for defining the heme-heme interaction constant n . The first set of four metal binding sites are easily titratable and show no interaction ($m=1$), whereas the second set of four titrate only at higher temperatures and have m values ranging as high as four at 40°. These results were taken to indicate temperature-induced conformational changes of the Hb chains, and it would appear that —SH groups may be related to the function of the protein. Indeed, Riggs has identified the oxygenation-linked proton-liberating groups responsible for the Bohr effect with appropriately placed —SH groups (147, 148) located in the β -chain of HbA (149) on the basis of a proportionality between the magnitude of the Bohr effect and the number of mercury ions bound by the native protein from numerous mammals. This has been disputed by Benesch & Benesch

(150), who reported that two reactive —SH groups of human Hb can be blocked without affecting the Bohr effect. —SH groups had earlier been considered to be involved in heme-heme interactions since blocking with *p*-chloromercuribenzoate decreases n (151, 152). The same author, however, has recently stated (148) that a mechanism of heme-heme interaction which requires —SH groups cannot be correct since similar values of n are found for mammalian hemoglobins having very different numbers of immediately available —SH groups.

Keilin (153) has recently reviewed the chemical and physicochemical evidence relevant to the nature of the heme iron-binding groups in Hb. These groups must be potential hemochromogen-forming groups (primary amino or imidazole). In the case of myoglobin (Mb), this conclusion is substantiated by the x-ray analysis (154) which has revealed the presence of a flat group which has dimensions compatible with an imidazole side chain of histidine and is co-ordinated with the heme iron atom from the protein side. By analogy, a similar situation most probably prevails in Hb (155), thus eliminating —SH (147) and carboxyl groups (156) from consideration (153). Hypotheses as to the function of these groups in heme-heme interaction or the Bohr effect must therefore consider that the heme iron co-ordinated groups are not the only ones involved. Suggestive of some possible function is the presence of two —SH groups in horse Hb which are readily available to heavy metals in the native protein, are located near two of the heme iron co-ordinated groups, and have the thiol side chain pointing in roughly the opposite direction to the presumed iron-linked imidazole (155). Heme-heme interaction and Bohr effect are not necessarily concomitant variables. Thus, Manwell (157) found that some invertebrate muscle and neurohemoglobins showed significant heme-heme interactions, although none of the proteins studied had a Bohr effect.

Murayama (158) has found that 2-methylnitrosobenzene binds HbA 10 times less strongly than nitrosobenzene, suggesting that the heme groups are to some extent buried in the protein in conformity with the results of a study of the relaxation time of water protons in methemoglobin solutions (159). The physicochemical evidence for the positions of the hemes in relation to the protein in Hb is discussed by Keilin (160) and George & Lyster (161, 162). Earlier electron-spin resonance results on the orientation of the heme groups were obtained by Ingram, Gibson & Perutz (163); the exact location of the hemes was finally decided by x-ray crystallography (155).

Wang and co-workers studied the combination of CO with Hb (164) and heme (165) and concluded that the fundamental property of Hb, the reversible combination of the ferrous heme with O₂ (oxygenation) without a subsequent oxidation of the iron to the ferric state, is attributable to the low dielectric constant of the medium in which the free or reactive side of the heme iron is located. This led to the making of a synthetic model Hb (166, 167) in which a ferrous heme ester co-ordinated on one side with an imidazole derivative is imbedded in a polystyrene film, leaving one iron valency free in the

low dielectric environment of the polymer. Such films oxygenated reversibly and showed the expected spectral variations.

The extensive and brilliant work of the group at Cambridge, England, on the x-ray crystallography of hemoglobins and myoglobins has recently led to three-dimensional Fourier syntheses of sperm whale Mb at a resolution of 2 Å units (154) and of horse Hb at a 5.5 Å resolution (155). These major landmarks in our knowledge of the structure of proteins are reviewed elsewhere (168). They will not be considered in detail here, except to point out that with Mb the resolution was sufficient to permit the identification of the amino acid side chains (169). This raises the probability that x-ray crystallography may be used to establish not only the spatial arrangement of the peptide chains of proteins but also the sequence of the amino acid residues. This may render chemical methods partly obsolete, particularly in terms of the requirements for finding long overlapping sequences to position peptides.

Multiple forms and primary structure.—Following the pioneer demonstration by Pauling and co-workers (170) that the blood of patients with sickle-cell anemia contained a hemoglobin that differed in electrophoretic mobility from HbA, the number of abnormal hemoglobins found increased rapidly, and today more than 30 have been described from human sources (171, 172, 248). Earlier work has been reviewed by Itano and others (173 to 176); discussions and more recent listings will be found in various journals (16, 171, 172; 177 to 180; 248). The presence of abnormal hemoglobins is genetically controlled (181 to 185; 203). Their separation and differentiation (186) has been based mainly on electrophoretic methods (174, 187), column-chromatographic methods (188 to 190), and differences in solubility (174, 191). Similar techniques have also revealed that normal adult human blood, in addition to its main component, also contains much smaller proportions of more than six minor Hb components (173; 190 to 201). With the exceptions of HbA₂ and HbF, which have β -chains that differ radically in amino acid sequence and are under separate genetic control (see below), it is uncertain whether these minor components are true genetic variants or simply artifacts of the main hemoglobin (201). More than one Hb has also been found in the blood of several animal species (131). It is still not known if this situation is peculiar to Hb or is more general, in the sense that genetically controlled differences in the amino acid sequences or other features of proteins with identical functions will be found in other cases. The presence of more than one "normal" naturally occurring form of some enzymes has been claimed (see 202). The variations of human haptoglobins and transferrins are under genetic control (204).

The first clear-cut demonstration that Hb consists of two equal halves was by x-ray crystallography (205). Symmetry was further established when it was found that complete digestion of HbA with trypsin gave rise to only half the number of peptides that would be expected from the total number of lysyl and arginyl residues in the protein (206) and that there were, contrary to previous reports, only four N-terminal valyl residues (207, 208)

α -chain: Val. Leu. Ser. Pro. Ala. Asp. Lys. Thr. Asp. Val. Lys. Ala. Ala. Try. Gly. Lys. Val.
10
Gly. Ala. His. Ala. Gly. Glu. Tyr. Gly. Glu. Ala. Ala. Leu. Glu. Arg. Met. Phe.
20 30
Leu. Ser. Phe. Thr. Pro. Thr. Lys. (His₂, Asp, Thr, Ser₃, Glu, Pro, Gly, Ala, Val,
40
Leu, Tyr, Phe₂). Lys. Gly. His. Gly. Lys. Lys. (His₃, Asp₆, Thr, Ser₂, Pro, Ala₇,
60
Val₃, Met, Leu₄). Lys. Leu. Arg. Val. Asp. Pro. Val. Asp. Phe. Lys. Leu. (Lys,
90 100
His₃, Asp, Thr₃, Ser₄, Glu, Pro₃, Ala₆, CySH, Val₄, Leu₇₋₈, Phe₂). Thr. Ser. Lys.
Tyr. ArgCOOH.

HbA that contains two α - and two β -chains can be symbolized as $\alpha_2^A\beta_2^A$. The normal variants carry instead of the β^A chain other peptide chains differing from it in at least several amino acid sequences. Thus, normal HbF has the constitution $\alpha_2^A\gamma_2^F$ (220 to 222), the N-terminal sequence of the γ -chain being Gly. His. Phe. (216), whereas the normal minor component HbA₂ (192) has the constitution $\alpha_2^A\beta_2^{A_2}$ (223). Various workers (174, 191, 224, 225, 226) have discussed HbF. On the other hand, the abnormal hemoglobins that have been structurally defined to date either lack α -chains altogether, being formed from four normal β -type chains [HbH is β_4^A (227) and Hb "Bart's" is γ_4^F (228)], or arise by the substitution of a single amino acid for a different one in either the α - or β -chain (Table I). However, HbM, which tends to oxidize to methemoglobin much more readily than HbA, may conceivably

not fall in either of these categories, the difference apparently residing in the structural relationship of the hemes to the protein (229); whether an underlying change in the primary or tertiary structure is responsible for this abnormal reactivity is not known.

TABLE I
STRUCTURE OF HUMAN HEMOGLOBINS

CHAIN VARIANTS		
Hemoglobin	Constitution	References
A	$\alpha_2\beta_2$	230, 231
F	$\alpha_2\gamma_2$	220, 222
A _s	$\alpha_2\delta_2$	223
H	β_4	227
"Bart's"	γ_4	228

SINGLE AMINO ACID RESIDUE VARIANTS			
Hemoglobin	Amino acid sequence	References	Position of sequence
A	Val.His.Leu.Thr.Pro.Glu.Glu.Lys.	216, 232, 233	} N-terminal sequence of β -chain (residues 1 to 8)
S	Val.His.Leu.Thr.Pro.Val.Glu.Lys.	232 to 235	
C	Val.His.Leu.Thr.Pro.Lys.Glu.Lys.	236	
G	Val.His.Leu.Thr.Pro.Glu.Gly.Lys.	233	
A	Val.Asp.Val.Asp.Glu.Val.Gly.Gly.Glu.Ala.Leu.Gly.Arg	237	} Residues 18 to 30 of β -chain
E	Val.Asp.Val.Asp.Glu.Val.Gly.Gly.Lys.Ala.Leu.Gly.Arg.	237	
D	As yet unidentified defect in this peptide (No. 26)	238	
A	Ala(Val, Try, Gly) Lys. Val(His, Leu, Tyr, Gly, Glu, Ala) Arg	239 to 241	} Derived from α -chain; probably residues 13 to 31
I	Ala(Val, Try, Gly) Glu. Val(His, Leu, Tyr, Gly, Glu, Ala) Arg	239 to 241	
D	As yet unidentified defect in this peptide (No. 23)	238, 239	

The implications of these remarkable findings in terms of the genetic control of protein synthesis have been amply discussed (174; 242 to 245).

Dissociations and recombinations.—It has long been known that the heme of Hb can be separated from the apoprotein at acid pH values (129). However, it has recently been shown (246) that even at neutral pH a ferrihemo-protein (Hb and Mb) is in fact in equilibrium with the apoprotein and hemin. If the equilibrium constant involved can vary widely with different hemo-proteins, this could explain (247) why attempts at purification of some hemo-proteins, such as thyroid peroxidase, have been plagued by unexpected losses of activity. The reconstituted Hb formed on combining native globin with protohemin followed by enzymic reduction of the hemin to heme is identical in all of its physicochemical properties with the original protein (249 to 252). The reaction between hemin and globin is kinetically complex and involves the formation of an intermediate (253, 254). The properties of the unnatural products formed on combination of Hb-globin with mesohemin, deuterio-

hemin (251, 252, 255), and various hemins that lack free carboxyl groups, (256) as well as those of similarly reconstituted myoglobins (257, 258), have been investigated. The heme-globin equilibrium has also been studied by measuring the quenching of the fluorescence of 1-diethylaminonaphthalene-5-sulfonamide groups attached to amino groups on the surface of hemo- and myoglobin (259). All these studies have shown that, although the point of linkage of the prosthetic group essential for oxygenation is at the heme iron, the carboxyl-bearing and, to a lesser extent, the vinyl side chains grossly affect the stability of the conjugation and the reactivity of the heme. The attachment of hemes other than protohemin IX to the appropriate apoproteins does not necessarily result in a decrease of functional activity for all hemoproteins. Thus, a reinvestigation (260, 261) of the combination of various hemins with the apo-horseradish peroxidase showed that mesohemin and hematohemin give an enzyme 1.3 times more active than the original peroxidase. Type I isomers as well as coprohemin III form inactive compounds. Deuterohemin-apoperoxidase has half the activity of peroxidase, whereas diacetyldeuterohemin gives an inactive enzyme. Hemin-*a*-apoperoxidase is similarly largely inactive as a peroxidase, a catalase, or a cytochrome-*c* oxidase (261, 262). The spectroscopic properties of compounds of hemin-*a* with a number of different proteins have also been studied (263).

This is not the only form of dissociation that Hb undergoes. At pH values of 3.5 to 6.0 (264 to 267), in alkaline solution (pH 11.0), (231, 268), in 1 *M* sodium chloride (269) or in urea solutions (268), the molecule of Hb dissociates into two approximately equal portions which reassociate at neutral pH or lower ionic strength to give a hemoglobin which is indistinguishable by physical criteria from the original material (231, 270). Native human globin appears to have a molecular weight of half that of HbA and in the absence of salts dissociates further into units which probably represent the individual peptide chains (271).

When a mixture of two hemoglobins that have identical α -chains but different β -chains, such as HbA ($\alpha_2^A\beta_2^A$) and HbS ($\alpha_2^A\beta_2^S$), is taken to a dissociating pH, acid or alkaline, the molecules then reassociated following neutralization, and the two original hemoglobins isolated, it is found that the chains that were identical have exchanged between the two (230, 245, 267, 272, 273, 274). Such "hybridization" has commonly been followed by utilizing C^{14} -labelled hemoglobins. In the above example, if both chains of the HbA had contained a C^{14} -amino acid, the HbS isolated after hybridization would have radioactive α -chains but unlabelled β -chains. See Perlmann & Diringer (275) for a discussion of the earlier work. Recent evidence (276) suggests that at least at alkaline pH values the primary dissociation is not asymmetrical, as previously concluded (267, 272), but symmetrical, leading to the formation of identical subunits ($\alpha\beta$) which are, in turn, in equilibrium with a small amount (less than 10 per cent) of the completely dissociated individual chains (α and β), thereby enabling the exchange of identical individual chains. A study of the rates of denaturation of mixtures of fetal and

normal adult hemoglobins had previously indicated that in alkaline dissociation identical, rather than different, subunits are formed (277). This explanation of the mechanism of hybridization has nevertheless been disputed (278). The hybridization technique has become a powerful tool in the study of the structural relationships of different hemoglobins; it has, for example, shown that the α -chains of HbA, HbF, HbS, and HbC are interchangeable and presumably identical, whereas the β -chains are not (273, 274, 279), that HbH contains four β -chains identical to those of HbA (280), and that in HbI the defect is in the α -chains, whereas the β -chains are the same as those of HbA (279); more recently, it has been extended to the study of various inter-species systems, such as between HbA and rabbit Hb (276, 278).

The criterion of identity of chains used in these experiments for hemoglobins of one species is the ability of presumably identical chains to replace each other in recombination experiments. Although so far there have been no indications that anything but entirely identical chains can do so, some differences could conceivably be compatible with replacements that lead to hemoglobins not separable by the techniques employed. Eventually a similar approach may be possible for other proteins that contain more than one peptide chain.

CYTOCHROMES OF THE C GROUP

Although cytochrome-*c* was prepared by Keilin in 1930 (281), it was first crystallized by Bodo (282) from King penguin muscle only in 1955. Shortly afterwards, crystalline cytochromes-*c* were obtained by Hagihara *et al.* and other workers (283 to 288) from 12 different sources both in the reduced and oxidized states. These cytochromes-*c* contained 0.45 per cent iron, comparable with previous chromatographically purified preparations (289, 290). Paléus & Theorell (291) found that crystalline cytochromes-*c* were electrophoretically inhomogeneous. Their main fraction contained 0.435 per cent iron. Procedures for the preparation of reduced cytochrome-*c* free from reducing agents (292, 293) and for the extraction of cytochrome-*c* with salts (294) have been described.

The inhomogeneity of preparations of chromatographically purified cytochrome-*c* has also been adduced from the separation of two hemato-hemin fractions (294 to 297) following the splitting of the thio ether bonds binding the heme to the protein (298) by the silver salt treatment of Paul (299). It is difficult, however, to decide whether cytochrome-*c* as it occurs *in vivo* is indeed inhomogeneous. Electrophoretic differences could be attributable to the presence of partially "unfolded" molecules, and different hemato-hemins could result from oxidation or isomerization reactions occurring during the drastic silver salt treatment.

A complete spectrum of 0.45 per cent iron native horse heart cytochrome-*c* has been reported (300). Applying the observations of Keilin & Hartree (301) on the intensification and sharpening of the absorption bands of re-

duced cytochromes at liquid air temperatures in a glycerol-water medium, Estabrook (302, 303) has recorded the spectra of numerous cytochromes at -190°C . With cytochrome-*c*, the reduced α -band splits into three components (301), the β -band into nine. As the protein is denatured, this fine structure becomes less and less distinct (301, 303), whereas a hemopeptide or "core" of cytochrome-*c* containing the heme and only 11 adjacent amino acids showed a single broad symmetrical α -band (303). These multiple α -bands probably do not represent several protein species but may well be caused by the resolution of vibrational components of the electronic transition resulting in the α -band, which is profoundly affected by the conformation of the protein around the prosthetic group (303). A direct relationship between the conformation of the protein and the properties of cytochrome-*c* has been demonstrated by Margoliash *et al.* (304). Isolating increasingly denatured, chromatographically defined fractions, these authors showed that the properties peculiar to cytochrome-*c*, such as the enzymic activities, the lack of auto-oxidizability and of combination with CO, and the relative pH independence of the reduced spectrum, are consequences of the native configuration of the protein. Completely denatured cytochrome-*c* containing the entire peptide chain of the native protein lacked these properties and acted like an ordinary chemical hemochromogen. Partly denatured fractions showed intermediate properties. Nozaki (305) has reported that trichloroacetic acid-denatured cytochrome-*c* exists as a dimer in solution, Morrison *et al.* (306) found that a similar acid-denatured preparation, even though it partly maintained its ability to stimulate the oxidation of ascorbate by rat liver mitochondria, was completely incapable of sustaining oxidative phosphorylation. They suggest that cytochrome-*c* itself is directly involved in the energy-conservation step. The oxidation reduction potential of cytochrome-*c* (307) is another property that has been found to vary with either changes in the conformation of the protein (308 to 310) or on digestion with proteolytic enzymes (311, 312). On the whole, those changes that cause a decrease of enzymic activity are accompanied by a lowering of E_0 toward more negative values (310, 312).

Studies of the effects on cytochrome-*c* of various chemical reagents and proteolytic enzymes have been in part summarized by Minakami *et al.* (313). Acetylation approaching completion resulted in a loss of enzymic activity with no increase in reactivity with CO (314). This is the only modification of cytochrome-*c* reported so far in which these two properties have not varied concomitantly. Guanidination of a considerable number, but not of all, of the ϵ -amino groups of lysyl residues, did not affect the enzymic or the chemical properties (315). On iodination, however, the protein lost its enzymic properties, became auto-oxidizable, and combined with CO (316). Coupling with *p*-diazobenzenesulfonic acid resulted in a disappearance of the typical reduced hemochromogen spectrum and in a complete loss of enzymic activity and of the capacity to undergo reversible oxidation and reduction (316), in-

dicating the involvement of at least one imidazole group in the oxido-reduction properties of the protein.

Conversely to the studies cited above which indicate the importance of the conformation of the protein on the properties of the hemochromogen, Nozaki and co-workers (317 to 320) have shown that the state of oxidation or reduction of the hemochromogen appears to have an effect on the conformation: the reduced form of cytochrome-*c* was less readily digested by proteolytic enzymes than the oxidized form. These observations recall the much older results of Jonxis (321), who found that oxidized cytochrome-*c* spreads far more readily than reduced cytochrome-*c* into a monolayer at a water-air interface.

Considerable advances have been recorded in the study of the primary structure of cytochrome-*c*. Tsou (322) first isolated peptide fragments attached to the heme following proteolytic digestion, and Tuppy and his collaborators (323 to 327) have determined the amino acid sequences of such "hemopeptides" or "cores" from proteins of eight different species. See Paléus & Tuppy (327) for a list of these sequences. The features common to all of them are: two cysteinyl residues separated by two other residues; a His. Thr sequence adjacent to one cysteine; and a basic residue, lysine or arginine, adjacent to the other. These recurring similarities probably indicate definite requirements of amino acid sequence compatible with cytochrome-*c* function. Thus, Ehrenberg & Theorell (328), studying an atomic model of the "core," showed that the two cysteinyl residues were correctly spaced so that their —SH groups could be linked into thio ether bonds with the two vinyl side chains of protoporphyrin. If the peptide were folded into an α -helix, either the histidine imidazole or the lysine ϵ -amino group readily co-ordinated with the heme iron but not both simultaneously. Paléus *et al.* (329) came to a similar conclusion from a study of the physicochemical characteristics of the "core." Margoliash *et al.* (304), on the other hand, showed that in a model in which the peptide was unfolded both the imidazole and the ϵ -amino groups could be made to co-ordinate with the heme iron on both sides of the heme plane. Harbury & Loach (312), investigating the spectral properties and the effect of pH on the oxidation-reduction potential of the "hemopeptide" from horse cytochrome-*c*, concluded that the "core" may well have different structures in the oxidized and reduced forms, either of which may or may not be related to the structure of the native protein. Bodo (329a) reported some preliminary x-ray diffraction measurements on crystals of horse heart cytochrome-*c*.

Amino acid analyses of various cytochromes-*c* have been performed by a number of different laboratories (328; 330 to 333). Takahashi *et al.* (334) reported the separation of peptides obtained by digesting horse heart cytochrome-*c* with trypsin. Titani *et al.* (335), using hydrazinolysis, identified the C-terminal residue as glutamic acid, whereas Titani *et al.* (336) found

that a digestion with carboxypeptidase did not appreciably decrease the enzymic activity.

Recent physicochemical studies have included: a report of the optical rotatory dispersion of solutions of cytochrome-*c* (337); a study of the chromatographic behavior of cytochrome-*c* on celite ion-exchange resins (337a); discussions of the "crevice structure" in which the heme is held (162) and the physicochemical parameters affected by such structures in terms of the pH dependence of cytochrome-*c* reactions, such as with cyanide (338) and azide (339); the determination of accurate sedimentation coefficients for mammalian cytochromes-*c* (340); and the synthesis of crystalline cytochrome-*c* heme, an adduct of heme, and two molecules of cysteines (341). It is not quite certain whether in cytochrome-*c* and the synthetic product, the attachment of the cysteine sulfur atom is to the alpha- or beta-carbon of the vinyl side chains (341 to 343), although it is most probably to the alpha-carbon.

Earlier work on the biosynthesis of cytochrome-*c* has been summarized by Drabkin (344) and by Vannotti (345). Marsh & Drabkin (346) and Yčas & Drabkin (347) studied the incorporation of labelled amino acids into the heme and protein moieties of cytochrome-*c* in rats, in a number of rat tissues *in vitro*, as well as in yeast cells adapting to oxygen; they showed that aerobic tissues possess independent capabilities for the complete synthesis of the hemoprotein.

The mode of attachment of cytochrome-*c* to the enzymes with which it reacts has been investigated. Smith & Conrad (348a, 348b) found that cytochrome-*c* itself as well as other basic proteins such as salmine, inhibited cytochrome-*c* oxidase. Beetlestone (349) observed similar phenomena in the reaction of cytochrome-*c* with yeast cytochrome-*c* peroxidase. Estabrook (350) studying the restoration by cytochrome-*c* of the succinic oxidase activity of particulate preparations from cytochrome-*c* deficient heart muscle, showed that inorganic cations, but not anions, competed reversibly with the hemoprotein, presumably for a specific locus on the particles. Jacobs & Sanadi (351), using dilute salt solutions, were able to extract practically all of the cytochrome-*c* present in rat liver mitochondria, while keeping the mitochondria relatively intact. Such depleted mitochondria took up the same amount of cytochrome-*c* as had been removed to restore maximal oxidation rates with a variety of substrates, as well as 75 percent of the original phosphorylating activity. All these experiments are consistent with the view that at least part of the forces binding cytochrome-*c* to the enzymes with which it reacts are electrostatic forces similar to those operative on cation exchanger resins. Thus the report (352) that endogenous cytochrome-*c* in heart muscle particles is lipid-bound and will specifically restore the NADH₂-oxidase activity of organic solvent extracted preparations only as a water-insoluble "lipid-soluble cytochrome-*c*" cannot be reconciled with the observations

cited above, unless one assumes that dilute salt solutions will reversibly separate the protein from the lipid, and that the forces binding these two component are largely electrostatic in nature.

Cytochromes of the c group from microorganisms and plants.—The realization that not only aerobic tissues but also organisms that are facultative or even obligate anaerobes contain cytochrome systems has, over the last decade, led to the isolation of a considerable number of *c* type cytochromes from such microorganisms. Among these may be listed *Chromatium* (353), *Desulfovibrio desulfuricans* (c_3) (354 to 357), *Chlorobium limicola* (358), *Chlorobium thiosulfatophilum* (359), *Rhodospirillum rubrum* (360 to 362), *Micrococcus denitrificans*, and *Pseudomonas denitrificans* (363), as well as the aerobic denitrifying organism, *Pseudomonas aeruginosa* (364, 365). One of the two cytochrome-*c* type heme proteins in this organism (366) has recently been crystallized (367). These cytochromes are placed in the *c* group because they all show spectra very similar or identical to those of the classical mammalian proteins. Nevertheless, their properties are commonly very different from those of the low molecular weight, highly basic proteins of the mammalian type. Some have acidic isoelectric points, and some have more than one heme per molecule; *Chromatium* cytochrome-*c* has, for example, three hemes for a molecular weight of 97,000 (353), whereas cytochrome- c_3 has two hemes for a molecular weight of 12,000 (356). The oxidation reduction potentials vary from +0.320 v. for *Rhodospirillum rubrum* cytochrome-*c* (360) to -0.204 v. for cytochrome- c_3 (356). Most of these cytochromes are inactive in mammalian enzyme systems; however, the cytochrome-*c* from *M. denitrificans* (363) appears in all respects to be identical with mammalian cytochrome-*c* and reacts with mammalian cytochrome oxidase. Relatively little is yet known about the structure of these proteins, although considerable information about their function in the metabolism of the microorganisms has accumulated. The properties and function of these pigments have been reviewed as related to anaerobic electron transport (368) and to photosynthesis (369, 370).

Atypical heme proteins which have properties different from those of any other class of heme proteins have been isolated from *R. rubrum* (371) and *Chromatium* (353). Kamen (372) has reviewed the properties and possible function of these "RHP" proteins, non-committally named from "Rhodospirillum heme protein."

Other cytochromes-*c* have been isolated from the rust *Ustilago sphaerogena* (373), from *Bacillus megatherium* and *Bacillus subtilis* (374), from the alga *Porphyr a tenera* (375, 376), and from *Azotobacter vinelandii* (c_4 and c_6) (377, 378). These last three have recently been crystallized (379 to 381).

Higher plants contain cytochromes-*c* similar to the mammalian proteins (382), such as the wheat germ cytochrome-*c* (383). Cytochrome-*f*, another variant of cytochrome-*c*, which was first detected (384) and prepared from green tissues of higher plants (385, 386), has more recently been isolated from *Euglena* (387) and a wide variety of algae (388).

The rapidly increasing number of cytochromes of the *c* group, as well as those of other groups, has led to some difficulties in the naming of new cytochromes, since knowledge of the obvious spectral properties has usually preceded the isolation of the protein and the study of its physicochemical properties. Moreover, an exact understanding of the function of these heme proteins is still lacking in many cases, thus precluding a functional classification similar to that used for enzymes. A widely used system (389) names the cytochromes according to the species or material of origin and the wavelength of the reduced α -band maximum. Egami *et al.* (390) proposed to name the cytochromes according to the nature of the heme group, the species of origin, and the oxidation-reduction potential. The Committee on Nomenclature of the International Union of Biochemistry is working on a generally acceptable system.

Morton (391) has recently compiled a classification of cytochromes. Several reviews exist on mammalian cytochromes (392 to 395) and on plant cytochromes (396 to 399).

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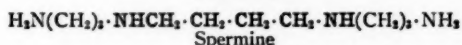
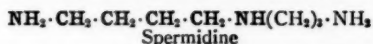
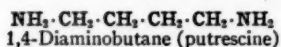
THE BIOCHEMISTRY OF THE POLYAMINES: SPERMIDINE AND SPERMINE¹

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Spermidine and spermine are aliphatic polyamines that are widely distributed in biological material. Although these amines have been known for many years² as normal biological constituents, it is only recently that much additional knowledge has become available on their involvement in various biological systems. We still do not know the specific function of these amines, but considerable information has recently been accumulated on their biosynthesis and metabolism, as well as on a variety of physiological and pharmacological effects. The possible physiological importance of these amines is perhaps best indicated by studies on their activity as growth factors for certain microorganisms and by recent studies on the relationship of these amines to nucleic acids and to problems of membrane stability.

Structurally, spermidine and spermine can be considered to be derivatives of 1,4-diaminobutane, as indicated in the following formulae:



ASSAY TECHNIQUES

Studies on these amines have been hindered by inadequate analytical tools. All of the earlier studies,² as well as some of the more recent investigations have depended on extraction and isolation of the amines; these procedures were necessarily time-consuming and inadequate for the accurate estimation of small quantities. For this reason, chromatographic techniques have been introduced. These represent a marked improvement but are still not completely satisfactory for the rapid estimation of these amines in many samples. For quantitative estimations, ion-exchange procedures (130, 150, 157) have been described. These methods involve the adsorption of the amines on such acidic adsorbents as Amberlite XE-64 or Dowex 50 and subsequent elution by salts or acids. The concentration of amines in the eluates is then determined by some non-specific assay, such as the dinitrofluorobenzene procedure. The specificity of this method depends on the chromato-

¹ The survey of the literature pertaining to this review was concluded in November, 1960.

² Most of the publications prior to 1950 have been reviewed in reference 61.

graphic position, since these polyamines are adsorbed more strongly than the amino acids and the common tissue constituents. In general, the specificity is not complete; to increase the specificity of the assays, these chromatographic methods should be combined with other procedures that have been used for the isolation and determination of spermidine and spermine. Some of these are: (a) preliminary alkaline extraction into *n*-butanol or *t*-butanol to separate the amines from the compounds containing acidic groups (109, 130); (b) preliminary distillation from an alkaline solution (65, 163); (c) successive chromatographic separations with different adsorbents; (d) calculation of the ratio (38) of the optical densities of the dinitrophenyl derivatives at 350 $m\mu$ and at 390 $m\mu$ to distinguish between primary and secondary amines (108); (e) paper chromatography with ninhydrin spray for detection (2, 24, 39, 47, 50, 65, 99a, 122, 123, 136a), (f) paper electrophoresis (26, 49, 50, 65, 163, 170); and (g) bioassay (65, 124) and bioautography (65). A color reaction of the polyamines with copper carbonate has also been used (8, 46, 48, 54, 112a, 125, 160) but is probably not sufficiently sensitive and specific for most purposes. In some experiments the use of these assays has been greatly facilitated by the addition of isotopic markers of C^{14} -diaminobutane, C^{14} -spermidine, or C^{14} -spermine (for synthetic procedures, see p. 584).

In summary, there are now a number of techniques that permit the satisfactory separation of spermidine and spermine and their derivatives from other known compounds in biological materials. These procedures, however, are still time-consuming, and no individual procedure is suitable as a general method unless combined with other techniques.

DISTRIBUTION AND CONCENTRATION

Mammalian tissues.—The distribution and concentration of spermidine and spermine in animal tissues have been studied by a number of investigators, who have used a variety of analytical procedures. The spermidine and spermine concentrations obtained by quantitative column chromatography have been tabulated by Rosenthal & Tabor (130). The accumulated data from various laboratories show that spermidine and spermine are widely distributed in animal tissues (50, 56, 62b, 74, 99a, 130, 163, 170). Considerable variations have been found in different tissues and different species, but particularly high concentrations have been reported in pancreas, prostate, and human semen. The high concentration of spermine in human semen (3.3 mg. per gm.) is mostly in the seminal plasma and is not related to the concentration of spermatozoa; it seems likely that the spermine of the semen originates in the secretions of the prostate (26, 44, 62, 62b).

Closely related to studies on the concentration of spermidine and spermine are studies on the diamines: 1,3-diaminopropane, 1,4-diaminobutane, and 1,5-diaminopentane. The concentration of these amines in animal tissues is not well-established, mainly because of a lack of sufficiently specific assays. Although Rosenthal & Tabor (130) did not find these compounds in most tissues, 1,4-diaminobutane has been reported in hog pancreas (50), calf

pancreas (50), ox lung (50), steer liver (163), possibly in rat spleen and mouse urine (130), and in human semen (163). 1,3-Diaminopropane has been found in steer liver (163) and human semen (163) but not in several other tissues (50). 1,5-Diaminopentane was recently reported in liver by Zillig *et al.* (170).

No definitive experiments have been reported on the intracellular distribution of spermidine and spermine. It is not possible to obtain this information by conventional separation of the nuclei, mitochondria, and microsomes by differential centrifugation, since the high affinity of spermidine and spermine for phospholipids and nucleic acids (84, 124) causes a secondary redistribution of the amines after homogenization (146). Zillig *et al.* (170) have reported that 1,5-diaminopentane and spermidine are present in liver ribosomes, but the possibility of a secondary redistribution was not excluded.

In contrast to bacteria (see below), no derivatives of spermidine or spermine have been described in animal tissues, except for the recent reports of Kósaki *et al.* (88 to 98c) that spermine was found in a new phospholipid, "malignolipin," which was isolated from malignant tissues. The isolated material was hygroscopic and has not been completely characterized. It had a nitrogen:phosphorus ratio of 5 and was basic. After hydrolysis an unidentified fatty acid was obtained, and paper chromatography indicated the presence of choline and spermine. Further work is needed on the identification of this material, as well as a rigid proof of its purity.

Kósaki *et al.* have published a large number of studies on the distribution of "malignolipin" and have reported that it is only present in malignant tissues. However, the methods used depend on rather involved extraction procedures, followed by relatively non-specific colorimetric assays, and it is essential that these results be checked by other methods before they can be adequately evaluated.

Microorganisms.—Large concentrations of spermidine and spermine, as well as 1,3-diaminopropane and 1,4-diaminobutane, have been found in a variety of microorganisms (32, 50, 59, 67, 157, 163). *Escherichia coli*, for example, contained 6.2 μM of 1,4-diaminobutane and 2 μM of spermidine per gm. of wet cells. With *Azotobacter vinelandii*, 1,4-diaminobutane (9 μM per gm.) and spermidine (4.4 μM per gm.) accounted for 4 per cent of the total cellular nitrogen and 22 per cent of the cellular non-protein nitrogen (157).

Herbst *et al.* (67) made the interesting observation that the polyamines were present in highest concentration in Gram-negative bacteria. Much lower concentrations were found in Gram-positive bacteria, and many Gram-positive species had little or no detectable amounts of these amines. Low, but definite, quantities of spermidine and spermine were also reported in several yeasts (50, 59, 67, 157).

During the past year, acetylated derivatives of 1,4-diaminobutane, spermidine, and spermine have been described in both *E. coli* (39) and *Staphylococcus aureus* (128). *E. coli*, grown on a purified medium, contained monoacetyldiaminobutane and two isomers of monoacetylspermidine, in addition to 1,4-diaminobutane and spermidine. Increase of pH or addition of triethanolamine or spermidine to the medium not only changed the total

quantities of 1,4-diaminobutane and spermidine in the cells but also increased the fraction of each amine present as the acetyl derivative. Addition of spermine to the medium resulted in even more marked changes; in this case, the cells mainly contained monoacetylspermine, diacetylspermine, and spermine.

Staphylococcus aureus, grown on a purified medium, contained only small amounts of polyamines (67, 128). In the presence of added spermine, both free and conjugated spermine were found in the cells. With added 1,4-diaminobutane, no amine was found in the cells, but high concentrations of monoacetyldiaminobutane were found in the medium (128).

E. coli also contained a derivative of spermidine, which released spermidine, glycine, glutamic acid, and cysteic acid (1:2:2:1) upon acid hydrolysis [Dubin & Rosenthal (37, 40)]. These data were tentatively interpreted as indicating a conjugate between glutathione and spermidine, although the hydrolysis data were not completely in agreement with that expected for glutathione. Recent work by Viswanatha & Rosenthal (unpublished) has indicated that this compound is probably not a simple spermidine-glutathione conjugate and that further work is necessary before the exact structure can be elucidated. When *E. coli* was grown in a medium containing spermine, a comparable conjugate containing spermine was found (37).

As indicated by the above experiments, the exact concentration of polyamines and their derivatives in various microorganisms can be markedly affected by the composition of the culture media, as well as by other cultural conditions, such as pH (132, 157). It is particularly important that the uptake of amines from the medium (39, 121, 124, 128, 163) be carefully considered in evaluating the amine content of bacteria grown in crude media, since these media may contain either amines or their precursor amino acids. It is possible, for example, that this accounts, at least in part, for the report of Zillig *et al.* (170) that *E. coli* ribosomes contained 1,5-diaminopentane, since this amine was not present in ribosomes of *E. coli* grown on a purified medium in the experiment of Cohen & Lichtenstein (32) (see below).

Relatively little is known about the intracellular distribution of the polyamines in the microbial cell, except for the recent work of Zillig *et al.* (170) and Cohen & Lichtenstein (32). By use of paper chromatographic and paper electrophoretic techniques, Zillig *et al.* showed that ribosomal particles from *E. coli* contained 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, and, possibly, spermidine; enough amines were present to neutralize one-third of the phosphate groups of the ribonucleic acid in the particles. Some evidence was presented to show that these amines were present in a complex with amino acids, RNA, and some unknown components within the ribosomes. However, as in liver (see p. 581) no experiments were reported to rule out possible artifacts caused by secondary redistribution of the amines during preparation of the extract.

In similar studies, Cohen & Lichtenstein (32) showed that 12 to 15 per cent of the total polyamines in an extract of *E. coli* B was in the ribosomal fraction, and, as indicated below (see p. 594), they presented evidence that

these amines were concerned with the stability of the ribosomal particles. The ribosomal polyamines did not exchange with labeled polyamines present in the remainder of the extract, and, thus, in these experiments the authors appear to have ruled out secondary redistribution of the amines.

Viruses.—High concentrations of 1,4-diaminobutane and spermidine have been reported in the bacteriophages T2, T4, and T6 of *E. coli* B [Ames, Dubin & Rosenthal (6, 7)] and bacteriophage 3 of *E. coli* 518 [Kay (82)]. The concentration of polyamines and of acetylated polyamines in the phage reflected the concentration of these amines in the host bacteria during the formation of the phage. A cation analysis of T4 bacteriophage showed that 1,4-diaminobutane and spermidine accounted for about 40 per cent of the cations present (6). 1,4-Diaminobutane and spermidine appear to be the two unknown nitrogenous compounds previously described in bacteriophage T2 by Hershey (69). Hershey also reported that these compounds were injected, along with the DNA, when the phage infected fresh cells.

The polyamines in these phages did not exchange with C^{14} -diaminobutane added to the crude bacterial lysate. This experiment ruled out an artifact caused by a secondary adsorption of the polyamine to the virus after lysis and indicated that the polyamines became associated with the phage during phage production.

In contrast to these data, no polyamines were found in a permeable mutant of *E. coli* B bacteriophage T4 (25), in bacteriophage T3, or in *Salmonella typhimurium* bacteriophage P22 (6). This was presumably because of the increased permeability of these phages and the consequent displacement of the polyamines by the Mg^{++} of the suspending fluids used during the isolation of the phages. However, a high spermidine concentration was obtained with the permeable mutant of bacteriophage T4 if the purified phage was resuspended in a spermidine-containing solution and then washed with water instead of Mg^{++} (6). (See pages 593–94 for a review of the effects of polyamines on the stability of *E. coli* bacteriophages.)

No significant amounts of polyamines were found in tobacco mosaic virus, cucumber virus, tomato bushy stunt virus, or polio virus, but no data were presented on possible losses during isolation of the viruses (6).

Miscellaneous.—Spermidine and diaminopropane have been found in the poisonous secretions of bird spiders (*Pamphobeteus tetracanthus*). The amines were combined with phenolic acids and were released by alkaline hydrolysis [Fischer & Bohn (49)]. Unbound spermine was reported by Ackermann & Janka (3) in the invertebrate *Cionia intestinalis*; spermidine and diaminobutane were not found in this organism. Spermine, spermidine, and diaminobutane were among the compounds detected by Ackermann (1, 2) in pupae of the silkworm, *Bombyx mori*; the amount of spermidine decreased markedly on pupation. Spermine has been reported by Ogata & Komoda (118) to be present in the sea urchin, *Echinococcus mirabilis*.

Very recently, two plant alkaloids have been shown to contain a spermidine moiety. The first observation was that of Baumann and co-workers (13, 42), who showed that spermidine was present in palustrin, $C_{17}H_{31}O_2N_3$,

a crystalline alkaloid isolated from the horsetail, *Equiperdum palustre*; the spermidine could only be isolated after degradation of the alkaloid. A partial structure was presented in which all of the nitrogen of the alkaloid was contributed by the spermidine moiety. Similarly, Potier *et al.* (119) isolated spermidine after alkaline degradation or acid hydrolysis of lunarine, $C_{25}H_{31}O_4N_3$, a crystalline alkaloid obtained from the seeds of *Lunaria biennis*. The spermidine was identified by several procedures, including gas chromatography and proton magnetic resonance spectra, and accounted for all of the nitrogen in the parent alkaloid.

CHEMICAL SYNTHESIS OF SPERMIDINE AND SPERMINE

Spermine was first synthesized by Rosenheim (127) and by Dudley, Rosenheim & Starling (41). More recently, the synthesis of spermine has been reported by Kormendy & Horváth (87) and Schultz (136). The synthesis by Schultz involved the following steps: (a) reduction of succinonitrile to 1,4-diaminobutane; (b) cyanoethylation of 1,4-diaminobutane with acrylonitrile to form N,N'-bis(2-cyanoethyl)diaminobutane; and (c) reduction of the latter compound to give spermine. Diacetylspermine and monoacetylspermine have been prepared by treatment of spermine with acetic anhydride (12). The synthesis of various analogues of spermidine and spermine has been described by Terent'ev *et al.* (159).

Spermidine was first synthesized by Dudley, Rosenheim & Starling (41) and more recently by Braun & Pinkernelle (23) and Danzig & Schultz (34). The Danzig-Schultz synthesis involved the steps: (a) treatment of γ -aminobutyronitrile with acrylonitrile to give N-(2-cyanoethyl)- γ -aminobutyronitrile; and (b) reduction of the latter compound to form spermidine. A modification of this synthesis with a higher yield was reported by Jackson (76). This synthesis involved the reduction of N¹(2-cyanoethyl)-N⁴-acetyl-1,4-diaminobutane to monoacetylspermidine and deacetylation by hydrochloric acid. The monoacetylspermidine prepared as an intermediate in this synthesis represents one of two possible isomers; the other isomer contains the acetyl group on the other primary amino group. This isomer has been prepared by treatment of spermidine with acetic anhydride and subsequent chromatographic separation on Dowex 50 (39).

C¹⁴-Spermidine and C¹⁴-spermine have been prepared by the reaction of 1,4-diaminobutane with acrylonitrile (30, 77). The use of C¹⁴-acrylonitrile resulted in labeling of the propyl moiety of spermidine and spermine. To obtain the label in the butyl portion of these compounds, C¹⁴-diaminobutane was used as the starting material. A number of syntheses of C¹⁴-diaminobutane have been described (30, 36, 157), and this compound is now commercially available. C¹⁴-Spermidine and C¹⁴-spermine can also be prepared biosynthetically (see next section).

BIOSYNTHESIS OF SPERMIDINE AND SPERMINE

Incorporation studies with C¹⁴-N¹⁵-diaminobutane in growing *E. coli* and *Aspergillus nidulans* (155, 157) and with 2-C¹⁴-methionine in *Neurospora*

The enzyme that catalyzes the first step is very similar to the methionine-activating enzymes purified from liver (27) and yeast (113). The *E. coli* enzyme has been purified 1500-fold (158), but no evidence has been obtained for the involvement of more than one enzyme in this reaction. With the *E. coli* enzyme, as with the yeast and liver enzymes, isotope experiments (158) demonstrated that the orthophosphate was derived from the terminal phosphate of ATP³²; in all three preparations, there was no evidence that ADP was an intermediate.

The enzyme that catalyzes the second step, adenosylmethionine decarboxylase, has been purified some twentyfold from the sonically ruptured cells of *E. coli*. The product of the decarboxylation reaction has been isolated by Dowex-50 chromatography. This compound has also been synthesized recently by Jamieson (78) by a modification of the synthesis employed for the preparation of adenosylmethionine (11). Both the enzymatically prepared and the chemically synthesized materials were suitable substrates for the third step.

The enzyme that catalyzes the third step, propylamine transferase, has been purified about 1000-fold from the sonically ruptured *E. coli* cells. A partially purified enzyme could be obtained free of the enzymes that degrade methylthioadenosine. With this preparation, therefore, it was possible to demonstrate that both spermidine and methylthioadenosine were formed (158). In this reaction, the propylamine moiety is transferred from the sulfonium compound to the receptor amine and is thus comparable to the methyl transfers previously described from adenosylmethionine.

No data have been presented on the enzymatic biosynthesis of spermine, although the isotope data with whole cells suggest that the mechanism is probably comparable to that described above for spermidine. No work has been reported on the enzymatic biosynthesis of these compounds in animal tissues, except for a preliminary report (155) on a small incorporation of C¹⁴-N¹⁵-diaminobutane into the polyamine fraction of minced rat prostate.

OTHER ENZYMATIC PATHWAYS INVOLVING SPERMIDINE AND SPERMINE

Amine oxidase: animal and plant.—Spermidine and spermine are oxidized by beef and sheep plasma (9, 71, 72, 123, 152, 166), and a soluble amine oxidase has been purified 150- to 200-fold from beef plasma (152). In addition to spermidine and spermine, this preparation oxidized a variety of primary monoamines and long-chain diamines; tryptamine, epinephrine, and 5-hydroxytryptamine were not attacked. Upon oxidation of the primary amines, the corresponding aldehydes and ammonia were obtained. The reaction products obtained upon oxidation of spermidine and spermine are still somewhat uncertain and will be discussed below. The oxidations were inhibited by cyanide and by isonicotinoylhydrazine (152). It is not known with certainty that a single enzyme is responsible for the oxidation of all of the substrates, although the available evidence supports this conclusion.

Blaschko & Hawes (20, 21) have reported a study of spermine oxidase activity in a variety of mammalian sera. Spermidine and spermine were

oxidized in the sera of all ruminants examined, e.g., camel, llama, giraffe, fallow deer, ox, sheep, and goat. Spermine oxidase activity was not found in any of the sera from the non-ruminants studied, although some of these sera were able to oxidize various primary monoamines (14, 18, 19).

The effect of age on the development of spermine oxidase activity was studied in the goat (20, 21). The ability to oxidize spermine was either absent or almost absent in the sera of newborn goats; the activity gradually increased during the first months after birth. In these experiments, the ability to oxidize benzylamine developed simultaneously with the spermine oxidase activity.

The plasma amine oxidase differed from the particulate amine oxidase of mammalian liver in its solubility, its substrate specificity, and its behavior toward inhibitors (152, 166). The liver enzyme oxidized a variety of monoamines but did not oxidize spermidine or spermine. It is possible that the polyamines do not have easy access to the enzyme site in the particulate preparations, but there is no evidence on this point at present. Spermine was not oxidized by a purified diamine oxidase preparation³ (80), although some activity has been reported with crude preparations from hog (169) and guinea pig (71, 73) kidneys. Thus, except for the sera of ruminants, there is only slight evidence for the enzymatic degradation of spermine in animal tissues *in vitro*. On the other hand, experiments *in vivo* with rats, mice, and rabbits indicated substantial degradation of spermine and the detection of 4 to 8 per cent in the urine as spermidine (130).

The oxidation of spermine by extracts of pea seedlings has been reported [Kenten & Mann (85)]; the rate of oxidation was approximately 8 per cent of the rate for 1,4-diaminobutane.

Amine oxidase: bacteria.—Spermidine and spermine are also oxidized by several bacteria. The organisms that have been shown to carry out this oxidation are *Pseudomonas pyocyanea* (139), *Pseudomonas aeruginosa* (120, 122), *Hemophilus parainfluenzae* (163), *Serratia marcescens* (122), *Mycobacterium smegmatis* (10, 131), *Pasteurella tularensis* (163), and *Neisseria perflava* (163, 164). Oxidation of spermidine and spermine could not be shown with a variety of other organisms, including *E. coli* and *Staphylococcus aureus* (122, 128, 139). In two cases, *P. aeruginosa* (122) and *M. smegmatis* (10), the kinetics of the oxidation by whole cells were reported and indicated an adaptive lag, which was not the case if polyamines had been added to the culture during growth.

In general, the oxidation of the polyamines by these bacteria has been studied with whole cells, freeze-dried cells, or cell-free extracts; purification of the enzymes involved has not been reported. Systematic comparisons of the various bacterial preparations with each other or with other amine oxidases have not been made. The available data do, however, indicate considerable differences in reaction products (see below) and substrate speci-

³ The oxidation of 1,4-diaminobutane and 1,5-diaminopentane by diamine oxidase is not included in this survey and has been reviewed elsewhere (153).

ficity. The *N. perflava* enzyme, for example, contrasted with the serum spermine oxidase in its apparent absolute specificity for polyamines. Neither monoamines nor diamines were oxidized, although a number of synthetic polyamines similar to spermine and spermidine were utilized [Weaver & Herbst (164)]. In general, the various preparations were all inhibited by semicarbazide, isonicotinoylhydrazine, and other carbonyl reagents (10, 122, 164).

*Reaction products of the enzymatic oxidation of spermidine and spermine.*⁴

The exact nature and stoichiometry of the oxidation of spermidine and spermine are not known. Different results have been found with preparations from different sources, and in no case has a complete stoichiometry been presented. There are several factors that make determination of an exact stoichiometry difficult. For example, it is very probable that an amino aldehyde is formed as one of the products during the oxidation. Such a compound would probably be volatile and would interfere with the determination of ammonia; furthermore, it would be very reactive and would undergo secondary changes. In most of the experiments, the results are further complicated by the use of whole cells or crude preparations with the resultant further metabolism of the primary products. Another possible complication of the use of whole cells is the effect of spermine in releasing other amines from the cell (39); this could easily lead to the erroneous conclusion that these released amines are intermediates. Another factor that should be considered in evaluating the data is the possibility that even a single enzyme may give multiple products by splitting the polyamine at alternate sites that have similar configurations (such as on either side of a secondary amino group, e.g., at C or B of Figure 2).

Even though the spermine oxidase of beef plasma has been partially purified, the exact nature of the reaction products is still very unclear. During the oxidation of spermine, two equivalents of O_2 (one equivalent when catalase was added) were consumed; two equivalents of ammonia and two equivalents of H_2O_2 were formed [Tabor *et al.* (152)]. These data indicate that the spermine molecule was oxidized at A and A' (Figure 2). However, the isolation of spermidine was also reported, as well as preliminary evidence for 1,4-diaminobutane and an unidentified amino aldehyde (149, 152). This would require splitting at C and at C' and is not consistent with the ammonia data. Furthermore, the oxidation of a secondary amino group would be necessary, and, as pointed out by Blaschko (18), other secondary amines are not oxidized by the serum enzyme. Recently, Bachrach & Bar-Or (9) have confirmed the above observation that spermidine is formed during the oxidation of spermine by serum amine oxidase. Using unfractionated sheep serum, they demonstrated by quantitative paper chromatography that all of the spermine could be accounted for by the spermidine that accumulated and that subsequently the spermidine was degraded further. The nature of the

⁴ See pages 597-99 for a survey of the papers concerned with the toxic effect of these oxidation products on bacteria, trypanosomes, spermatozoa, and renal tubules.

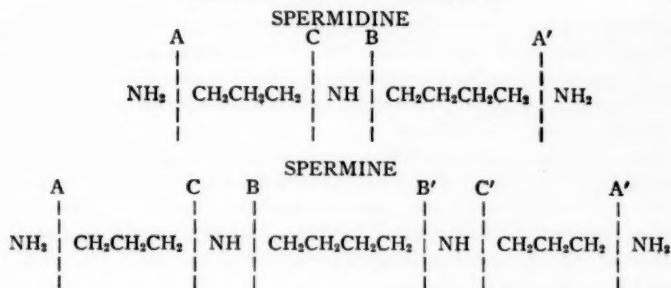


FIG. 2. Possible points of cleavage in the enzymatic degradation of spermine and spermidine.

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With *Serratia marcescens* (122) similar compounds were detected during spermidine oxidation. With this organism the somewhat surprising observation was made that, in contrast to spermidine oxidation, spermine was slowly metabolized, and oxygen was not consumed. More detailed quantitative data are necessary, however, to permit an adequate evaluation of this observation.

With *Mycobacterium smegmatis* cells (freeze-dried), the oxidation of spermine resulted in chromatographic evidence for the formation of β -alanine, 1,3-diaminopropane, γ -aminobutyric acid, and spermidine [Bachrach *et al.* (10)]. This is similar to the *Pseudomonas aeruginosa* oxidation (above) and would require splitting at B or B' as well as at C.

With the *Neisseria perflava* enzyme, 1 microatom of oxygen was consumed per μM of spermine or spermidine oxidized. One equivalent of H_2O_2 was formed but was decomposed by catalase in the extract. One μM of 1,3-diaminopropane was produced per mole of spermine or spermidine oxidized and was detected by a paper-chromatographic procedure [Weaver & Herbst (163)]. This would indicate a single oxidation at position B of spermine or position B of spermidine.

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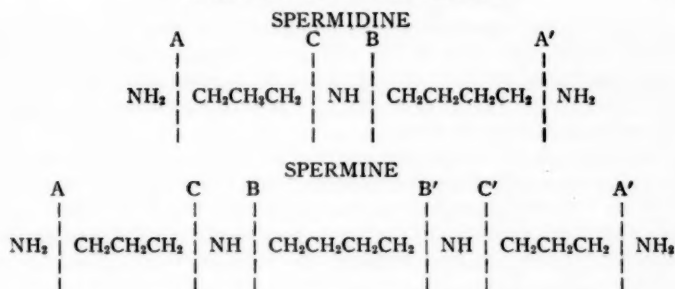


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Acetylation and conjugation.—As indicated in a previous section (pp. 581–82), recent work with *E. coli* and *Staphylococcus aureus* has demonstrated that spermine and spermidine can form acetyl and “glutathione” derivatives (37, 39, 128). Enzymatic studies on the formation of these derivatives have not been reported.

Transglutaminase.—A soluble enzyme which has been found in many mammalian tissues catalyzes the exchange of the amide group of certain proteins with a variety of amines, including spermine and 1,4-diaminobutane; ammonia is liberated from the amide groups. This enzyme has been partially purified from guinea pig liver and has been shown to require Ca^{++} (30, 31; 114 to 117; 134).

MICROBIOLOGICAL REQUIREMENTS FOR POLYAMINES AS GROWTH FACTORS

Although the physiological role of spermidine and spermine is still not known, the possible significance of these amines is perhaps best shown by their activity as growth factors for certain microorganisms. The experimental work on the amines as growth factors can be separated into: (a) studies on organisms with an absolute diamine or polyamine requirement; (b) studies on those organisms in which a stimulation of growth has been shown with these amines, without an absolute requirement; in some of these studies high salt concentrations were found to substitute for the amines; and (c) studies on the ability of the amines to antagonize the growth-inhibiting effects of quinacrine, quinine, diamidines, and proflavine.

Hemophilus parainfluenzae.—The first demonstration of a requirement of a microorganism for these polyamines was made by Herbst & Snell (66) with *H. parainfluenzae*. This organism grew on a purified medium only when 1,4-diaminobutane, spermidine, spermine, or agmatine was added. Maximum growth was obtained with concentrations of 0.1 μg . of 1,4-diaminobutane per ml.

Subsequently, Herbst *et al.* (64) extended these studies on this nutritional requirement of *H. parainfluenzae* and reported 20 compounds with growth factor activity; all of the active compounds were derivatives of 1,3-diaminopropane or 1,4-diaminobutane with at least one unsubstituted amino group. Several derivatives of 1,4-diaminobutane that were inhibitory were also reported, such as the N, N'-dialkyl and N,N,N',N'-tetraalkyl derivatives. At low levels these were reversed by added 1,4-diaminobutane, but constant inhibitor/growth-factor ratios could not be observed. A number of other compounds were inactive, including diaminoethane, 1,5-diaminopentane, and ornithine. Herbst *et al.* (67) have used *H. parainfluenzae* extensively in the bioassay of various growth factors.

Aspergillus nidulans.—Sneath (140) reported a mutant of *A. nidulans* that required 1,4-diaminobutane. There was no growth in the absence of 1,4-diaminobutane; maximum growth was obtained with 1 μg . per ml. Slight growth activity was obtained with spermidine (140), but none with spermine (151). 1,3-Diaminopropane and 1,5-diaminopentane were also completely

ineffective; when added with 1,4-diaminobutane, these amines were weak competitive inhibitors (140).

The low activity of spermidine and the inactivity of spermine is somewhat surprising in view of the observation that this mutant converted C^{14} - N^{15} -diaminobutane to spermidine and spermine even when it was grown on limiting amounts of the 1,4-diaminobutane (157). It should be noted, however, that permeability factors have not been excluded, and this may be of particular importance with the multivalent polyamines.

Neisseria perflava and *Pasteurella tularensis*.—Martin *et al.* (106) showed that the growth of *N. perflava* in a purified medium required 1,4-diaminobutane, spermidine, agmatine, or possibly 1,5-diaminopentane. These results were confirmed by Mager (101), who showed, however, that 0.1 *M* sodium chloride, potassium chloride, glucose, or sucrose could largely replace the polyamine requirement.

Similarly, Traub *et al.* (162) showed that *P. tularensis* required both sodium and spermine for optimal growth; the growth lag, observed on a chemically defined medium, was diminished by addition of spermine or spermidine (105). Mager *et al.* (101, 105) reported that with this organism also sodium chloride and spermine could be substituted for each other to a large extent, although maximal growth rates were only obtained when both factors were added together. With *P. tularensis*, as well as with *N. perflava*, it is likely that the polyamine acts by stabilizing the organisms (see pp. 592-95), rather than as a specific growth factor.

Fleming & Foshay (52), in a preliminary note, have reported that the addition of spermine, spermidine, 1,4-diaminobutane, 1,5-diaminopentane, or agmatine to a *P. tularensis* culture led to a twelve- to twentyfold increase in viable count, even though the dry weight increased only twofold. The organisms were considerably smaller, and the authors concluded that the effect of the amines was directed toward cell division rather than increased growth yields.

Lactobacillus casei.—During their studies on the effect of "strepogenin" in increasing the growth of *L. casei*, Kihara & Snell (86) noted that, even though some stimulation of growth was observed upon the addition of peptide fractions to the medium, the stimulation was greater when spermine or spermidine was added with these peptides. The polyamines, on the other hand, had no effect in the absence of the peptide fraction. *N*(3-Aminopropyl)-1,3-propanediamine was nearly as active as spermidine. 1,3-Diaminopropane and 1,4-diaminobutane had 0.001 of the activity of spermine, whereas 1,5-diaminopentane, agmatine, arcaine, histamine, tyramine, and tryptamine were inactive. These authors pointed out that contamination with spermidine and spermine probably accounted for part of the growth-promoting activity of crude "strepogenin" preparations, particularly since these were frequently derived from pancreas, which is a rich source of spermidine and spermine.

Achromobacter fischeri.—Spermine exerted a definite growth-furthering

effect when added to media containing amounts of NaCl that were osmotically inadequate for this obligate halophilic organism. This effect can be explained by the ability of spermine to prevent the lysis of these organisms that would otherwise have occurred in the absence of a high salt concentration [Mager (102); see also pages 593-95].

Antagonism to quinacrine, quinine, diamidines, and proflavine.—One of the earliest observations on the effects of polyamines in microbiological systems was the observation that spermine, spermidine, and several related synthetic polyamines overcame the growth inhibition that occurred when quinacrine (atabrine) or quinine was added to cultures of *E. coli*. (110, 139). Ca^{++} or Mg^{++} was also effective in antagonizing this inhibition (137, 138), but a number of monoamines and diamines were essentially inactive.

Similarly, low concentrations of spermidine and spermine overcame the inhibition of *E. coli* by propamidine (110, 141). Although spermine did not prevent the inhibition of *E. coli* and of *Staphylococcus aureus* caused by stilbamidine or pentamidine, this inhibition was prevented by high concentrations of diethylenetriamine, triethylenetetramine, and tetraethylenepentamine [Bichowsky-Slomnitzki (16)]. High concentrations of polyamines have also been shown to overcome the proflavine inhibition of an *E. coli* bacteriophage [Kay (82)]; in this case, 1,4-diaminobutane was the most active compound.

With intact *E. coli* and *S. aureus* cells, Bichowsky-Slomnitzki (17) also studied the diamidine inhibition of several dehydrogenase activities, respiration, and glycolysis and showed that this inhibition was reversed by triethylenetetramine and several other polyamines but not by spermine. With yeast cells, Massart (107) showed that spermine reversed the inhibition of respiration caused by trypanflavine, methylene blue, quinine, crystal violet, and protamine.

STABILIZING EFFECTS OF POLYAMINES

Bacteria: whole cells.—Closely related to the microbiological requirements for polyamines is the finding of Mager (101, 102, 103) that these substances have a marked stabilizing effect on certain bacteria that require a high osmotic environment for survival. As indicated in the previous section, it is likely that this stabilizing effect may explain some of the observations on the growth-promoting activity of the polyamines.

Mager (101) first demonstrated this stabilizing effect of polyamines in experiments with *Pasteurella tularensis*, in which he observed a decrease in viability when the cells were washed with distilled water instead of saline. Subsequently, he reported (103) that the cells "leaked" material absorbing at 260 μ when suspended in hypotonic media and that some lysis occurred. In the presence of chelating agents the lysis was markedly increased. These effects were prevented by the presence of microgram quantities of spermine, 1,4-diaminobutane, 1,3-diaminopropane, and diaminoethane (in order of decreasing effectiveness). Mager suggested that the excessive osmotic fragility of these organisms derived from an inherent deficiency of their cell walls (see the protoplast section, next page).

Mager also studied the prevention of osmotic lysis in *Achromobacter fischeri* (102). This organism was stable when suspended in 0.25 to 0.5 *M* NaCl, but lysed at lower concentrations of NaCl or in distilled water. The lysis of the cells could be completely prevented by 0.0005 *M* spermine. Spermidine and 1,4-diaminobutane were practically inert. Similarly, washed cells of *Staphylococcus aureus* died rapidly when suspended in distilled water. Addition of spermine, spermidine, 1,4-diaminobutane, or 1,5-diaminopentane diminished the rate of death [Razin & Rozansky (124)].

Related studies have been carried out by Herbst & Doctor (63) and by Keister (84) with *Hemophilus parainfluenzae*, which requires polyamines for growth. Although there was no loss of viability when this organism was suspended in polyamine-free media, there was a considerable loss to the suspending solution of material absorbing at 260 μ . At the same time, the RNA, but not the DNA, of the cells decreased. The leakage material reacted with orcinol and was considered to be composed of RNA breakdown products. Addition of spermine, spermidine, or, to a lesser extent, 1,4-diaminobutane or 1,3-diaminopropane markedly decreased the leakage of this material. Herbst & Doctor (63) and Keister (84) attributed this effect of the polyamines to an inhibition of the RNAase of the cell (see page 600).

Protoplasts.—Recently, as a development of Mager's earlier work with whole cells, Mager (103) and Tabor (142, 143) showed a stabilizing effect of polyamines on *E. coli* protoplasts (spheroplasts). The protoplasts were prepared in sucrose-containing media by either the penicillin or lysozyme-ethylenediaminetetraacetate technique and lysed when diluted with water or hypotonic media. This lysis was prevented if the medium contained low concentrations of spermine, spermidine, 1,4-diaminobutane, or 1,5-diaminopentane. Ca^{++} and Mg^{++} were also effective (146), but no effect was seen with NaCl, KCl, NH_4Cl , L-lysine, or L-ornithine (142, 143).

In another series of experiments, it was shown that protoplasts would lyse, even in hypertonic media, when treated with unsaturated fatty acids. This lysis was also prevented by 10^{-3} *M* concentrations of spermine (144).

Bacterial cell walls.—Purified cell wall preparations of a Gram-negative marine bacterium underwent autolysis at pH 8; 10^{-3} *M* spermine inhibited this autolysis [Brown (25a)].

Mitochondria.—A related group of experiments involves the ability of polyamines to inhibit the swelling of mitochondria, as measured by changes in the optical density at 520 μ (68, 142, 143, 144). In one study, low concentrations of spermine or spermidine (10^{-3} to 10^{-4} *M*) inhibited swelling caused by hypotonic media, succinate, and unsaturated fatty acids but did not inhibit swelling caused by phosphate, ascorbate, or thyroxine [Tabor (142, 143, 144)]. In another study, 10^{-4} *M* concentrations of spermine or spermidine inhibited spontaneous swelling of mitochondria and swelling attributable to succinate, β -hydroxybutyrate, glutamate, thyroxine, calcium ions, phosphate, glutathione, ascorbic acid, arsenite, and oleate [Herbst & Witherspoon (68)]. 1,5-Diaminopentane and 1,4-diaminobutane were less effective than spermidine and spermine (68, 143). Mg^{++} was effective, as

had been previously shown (75), but NaCl, NH_4Cl , KCl, L-lysine, and L-ornithine had little or no effect (142, 143).

The mechanism of the protection of mitochondria against induced swelling is not known but possibly could be related, at least in part, to polyamine binding of the fatty acids that have been shown by Lehninger & Remmert (100) to be released by mitochondria. The observation that spermine inhibits swelling induced by added fatty acids is consistent with such a hypothesis (68, 144). These findings are similar to the effect of spermine in inhibiting fatty acid-induced lysis of protoplasts and rat erythrocytes (144).

Associated with the swelling of the mitochondria is a loss to the suspending medium of material which absorbs at 260 μ . This loss is partially inhibited by spermine (142, 143). Similarly, spermine and spermidine inhibited the leakage of such material from microsomes (142, 143).

Ribosomes.—Stabilization of ribosomes from *E. coli* has recently been reported by Cohen & Lichtenstein (32). These authors showed that the addition of spermidine, but not 1,4-diaminobutane, preserved ribosomal complexes, which otherwise tended to decompose into their component parts; the integrity of the ribosomes was measured by the sedimentation constant in ultracentrifugal analyses. Mg^{++} also preserved the ribosomes, but a greater effect was found with Mg^{++} and spermidine together. Cohen & Lichtenstein suggested that " Mg^{++} and spermidine are the naturally occurring clasps on the complex ribosomal components existing in the bacterium." This viewpoint is supported by the analytical data of Cohen & Lichtenstein (32) and of Zillig *et al.* (170), which showed that both 1,4-diaminobutane and spermidine were present in the ribosomes (see also pp. 580–84 of this review).

Bacteriophages.—Polyamines have also been shown to stabilize bacteriophage preparations. Fraser & Mahler (55) used urea-shocked bacteriophage (π) derived from *E. coli* bacteriophage T2. This preparation, in contrast to the parent T2, will infect only protoplasts and will not attack whole cells. Fraser & Mahler showed that the addition of 0.01 *M* 1,5-diaminopentane or spermidine will protect this shocked phage from inactivation caused by such procedures as dilution or heating. 1,5-Diaminopentane was the most effective polyamine in this system.

With *E. coli* bacteriophage T5, a stabilizing effect was obtained with extremely low concentrations of polyamines [Tabor (154)]. Spermidine (2×10^{-7} *M*) and spermine (2×10^{-8} *M*) were shown to protect this bacteriophage from the inactivation that occurred when the phage was diluted in media that contained citrate or ethylenediaminetetraacetate. 1,4-Diaminobutane and 1,5-diaminopentane were also effective but at higher concentrations (10^{-5} *M*).

The exact mechanism of this stabilization is not known, but, presumably, the polyamines replace the divalent cations that are removed by the chelating agent. This would probably result in a stabilization of the helical structure of the nucleic acids (see pp. 595–97). Data are not available on the relative importance of the polyamines and of the divalent cations in these stabilization effects or on the effect, if any, that the polyamines exert in the presence of excess Mg^{++} or Ca^{++} .

Transforming principle.—Spermine has also been shown to stabilize a transforming DNA preparation from *Bacillus subtilis*; in the presence of spermine, a higher temperature was necessary for inactivation (154a). When the DNA was heated in 0.001 *M* potassium dimethylglutarate, pH 6.2, for 30 min. periods, 50 per cent inactivation was observed at 73°; in the presence of 10^{-4} *M* spermine, a temperature of 91° was required for a comparable inactivation. Stabilizing effects were obtained with as little as 5×10^{-4} *M* spermine; stabilization was also observed with spermidine and 1,4-diaminobutane, but considerably higher concentrations were necessary.

Phosphatase and β -glucuronidase.—The acid phosphatase of seminal fluid loses activity upon dilution. This inactivation may be prevented if low concentrations of spermidine or spermine are present in the diluting fluid; addition of the polyamines after inactivation does not lead to restoration of the activity. A number of other compounds, including 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, albumin, and gelatin are also effective, but amino acids have little, if any, protective activity. The protective activity of the amino compounds depends on the amino groups, since treatment of these compounds with nitrous acid or with acetic anhydride destroyed the activity. Conversion of the primary amino groups to secondary amino groups by benzylation or dinitrophenylation did not destroy their protective activity [Jeffree (79)].

Studies with highly purified β -glucuronidase demonstrated that spermine protects against the loss of activity which results from dilution [Bernfeld *et al.* (15)]. Spermidine, 1,5-diaminopentane, 1,4-diaminobutane, and 1,3-diaminopropane were less effective. The activating potency of the compounds tested was attributed to the presence of two or more basic groups and the absence of any carboxyl groups. The mechanism of this activation is still not clear, although the authors suggested that the various effects could be attributed to a reversible dissociation of the enzyme.

BINDING OF POLYAMINES TO NUCLEIC ACIDS, LIPIDS, AND HEPARIN

Nucleic acids.—A number of papers have been published on the binding of polyamines to nucleic acids (6, 16, 27a, 45, 74a, 83, 84, 124). Although much of the evidence is indirect and little is known of the magnitude of the association constants, the data definitely indicate that polyamines and nucleic acids have a strong affinity for each other. Complexing of polyamines and nucleic acids was indicated by such observations as the formation of an insoluble polyamine-nucleic acid complex when dilute solutions of spermidine or spermine were added to nucleic acid solutions (84, 124); by precipitation of the complex from ruptured cells of *Hemophilus parainfluenzae* upon addition of ethanol (84); by dialysis studies (146); and by neutralization by nucleic acids of the antibacterial properties of spermine (16, 124). In general, similar results were found with ribonucleic acids and deoxyribonucleic acids. Complex formation was seen most clearly in distilled water and was reversed by a high salt concentration.

Recently, further evidence for a nucleic acid-polyamine complex was obtained by the demonstration of a hypochromic effect when polyamines were

added to solutions of nucleic acid or of oligonucleotides (45, 84). Keister (84) observed a decrease in the absorption at 260 $m\mu$ when spermine was added to an aqueous RNA solution. Felsenfeld & Huang (45) used mixtures of polyadenylate and polyuridylylate and showed that the optical density decreased when 1,4-diaminobutane, 1,5-diaminopentane, or 1,10-diaminododecane was added. All of the diamines were equally effective and appeared to be only slightly less effective than Mg^{++} . These results have been interpreted as indicating that Mg^{++} or the polyamines can facilitate the formation of a two-stranded complex. Although the mechanism of this effect is probably attributable to neutralization of the phosphate groups on the polynucleotides and consequent increase in the effectiveness of hydrogen bonding, other explanations, such as cross-linkage, have not been completely excluded.

In a preliminary study, Felsenfeld & Huang (45) also showed, by conductimetric titration, that there was a strong interaction between single-stranded polyuridylic acid and polyamines; spermine had a particularly high affinity. When spermine was added to a solution of barium polyuridylylate in distilled water, the spermine quantitatively displaced the barium and an insoluble spermine-polyuridylylate complex formed (45).

In a continuation of these studies, Huang & Felsenfeld (74a) demonstrated that the different spermine-polynucleotide complexes had markedly different solubilities. Polyadenylate and polyinosinate were precipitated by the addition of one equivalent of spermine per equivalent of polymer phosphate; polycytidylylate and polyuridylylate were not precipitated under these conditions but required excess spermine. The two-stranded poly (adenylic + uridylic) complex was completely precipitated by spermine, but the poly (inosinic + cytidylic) complex was not precipitated under comparable conditions (pH 5.1 to 6.8).

In a somewhat similar study, Cantoni (27a) demonstrated that a partial fractionation of "soluble RNA" could be obtained by differential precipitation of this RNA with spermine under carefully controlled pH and ionic strength. Fractions were obtained that were two to three times richer in the valine-specific and in the proline-specific soluble RNA fractions.

Although the exact physiological significance of the polyamine-nucleic acid affinity is not clear, studies of this binding may be relevant to a number of other observations, which are cited elsewhere in this review. Among these observations are the high concentration of polyamines in certain bacteriophage preparations; the passage of polyamines into the host cell together with the phage DNA; the high concentration of polyamines in ribosomes; the stabilization by polyamines of certain bacteria, protoplasts, ribosomes, bacteriophages, and transforming principle; the uptake of polyamines from culture media by both living and killed cells; the inhibition of RNAase and DNAase by polyamines; and the effects of polyamines on plant chromosomes.

Lipids.—Spermine has been shown to inhibit the cytochrome-*c*-catalyzed oxygen uptake of unsaturated fatty acids [Tabor (144)]. The mechanism of this inhibition is not known, but it is possible that it is caused by a binding of the fatty acids by spermine. In other studies, Razin & Rozansky (124)

have found that low concentrations of spermine, spermidine, diethylenetriamine, and triethylenetetramine form insoluble complexes with lecithin solutions. Complexes were not formed with 1,4-diaminobutane or 1,5-diaminopentane. Furthermore, lecithin prevented the antibacterial action of spermine (see page 598).

Here, as with nucleic acid, the significance of the affinity of polyamines and lecithin is not known. However, the high concentrations of phospholipids in the membranes of bacteria, protoplasts, and mitochondria suggest the possibility that this type of polyamine-phospholipid complex may account for the effects of polyamines on membrane stability (see pp. 592-95). In addition, it is probable that complexes of polyamines with phospholipids and with nucleic acids affect the concentrations of these amines in various cells and tissues.

Heparin.—The ability of heparin to complex with spermine, spermidine, and 1,4-diaminobutane was demonstrated by Amann & Werle (4) by the precipitate that developed when these amines were added to heparin in alcohol. Furthermore, the addition of spermine to a heparin-histamine complex increased the dissociation of the latter. Similar to these studies were the experiments of the same authors (5) on the decreased solubility of thrombin in water when spermine was present; this effect could be overcome by heparin addition. The complexing of spermine with heparin probably explains the clotting of heparinized blood induced by the addition of spermine [Tabor & Rosenthal (149)]. Binding of spermine by heparin was also indicated by the experiments of Mager (103) with protoplasts, in which heparin prevented stabilization by spermine.

PHARMACOLOGY AND PATHOLOGY

Animal studies.—Earlier work [Rosenthal *et al.* (51, 129) demonstrated that single injections of spermine (0.1 to 0.2 mM per kg.) in a variety of animals resulted in acute renal insufficiency and death within four to 10 days; this was caused by an acute necrosis of the epithelium of the proximal convoluted tubules of the kidneys. With sublethal doses permanent renal damage was not found, and, therefore, a chronic lesion could not be produced. Spermidine was only one-twentieth as toxic as spermine. In man, intramuscular administration of spermine caused albuminuria, hematuria, and azotemia [Risetti & Mancini (126)].

More recently, two methods of producing a chronic renal lesion in rabbits have been observed: (a) by repeated subcutaneous injections of spermine—producing fibrosis and narrowing of the renal cortex bilaterally; (b) by injection of spermine into the left renal artery—resulting in unilateral fibrosis and shrinkage of the entire left kidney. A number of these rabbits developed cardiac hypertrophy and hypertension in addition to the chronic renal lesions (147, 148).

Other studies on the pharmacological actions of spermine have revealed central nervous system depression (61), hyperglycemia (43, 126), an antihistaminic effect (99), little or no effect on the blood pressure (35), an acute diuretic effect (149), an antitumor effect (22, 111a), an antiheparin effect (5,

149), an anticoagulant effect in high concentrations (5, 149), and, when oxidized by beef plasma amine oxidase, a toxic effect on spermatozoa (149). Spermine has been shown to increase the motility of spermatozoa from mice, rats, guinea pigs, and rabbits but not from bulls or rams (149, 167). Spermine has also been observed (4) to cause a histamine-like contraction of the isolated guinea pig intestine; this was attributed to its action as a histamine-releaser.

A number of studies on the spermine content of the blood and cerebrospinal fluid in epilepsy (125, 160), in malignancy (8, 33, 54, 112, 161), and in pregnancy (112a, 136a) have been reported. Most of these studies were carried out with relatively insensitive and non-specific methods and will have to be repeated before their significance can be evaluated.

Recently, Kósaki *et al.* (88, 90, 95, 97) have reported that "malignolipin," the spermine-containing phospholipid (see page 581), may be isolated from the blood of cancer-bearing patients but not from the blood of non-cancer patients. Similar results have also been reported by Tago *et al.* (158a) and by Hara & Hasegawa (62a).

Microbial studies.—Various studies have been reported on the effect of spermine on microorganisms (16, 57, 60, 86, 121, 123, 124, 132, 133, 135, 149, 165). Some of the organisms for which spermine has an inhibitory effect are *Staphylococcus aureus*, *Staphylococcus albus*, *E. coli*, *Lactobacillus casei*, *Bacillus anthracis*, *Shigella flexneri*, *Corynebacterium hofmannii*, *Streptococcus hemolyticus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and various species of *Saccharomyces*, *Debaryomyces*, and *Rhodotorula*.

In general, spermine was most toxic, spermidine was somewhat less so, and 1,4-diaminobutane and 1,5-diaminopentane had no significant toxicity. With *Staphylococcus aureus*, *E. coli*, and *Saccharomyces cerevisiae*, the toxic effect of spermine was shown to be greatest in alkaline media and decreased when the pH was lowered (121, 132, 149).

The inhibition of *S. aureus* has been studied in greater detail [Razin & Rozansky (124)] and has been shown to be a bactericidal effect. This effect only occurred in a nutritionally adequate medium, thus indicating that satisfactory growth conditions were necessary; on the other hand, a decrease in toxicity was not noted when the temperature was lowered to 6°. The toxicity of spermine was inhibited by inorganic salts, by a number of other basic compounds, by nucleic acids, and by lecithin (16, 60, 124). It is likely that the antibacterial effect of spermine accounts for the antibacterial effect of human semen against *S. aureus* (62, 123).

The polyamines also have an inhibitory effect on bacteriophages. Spermine and spermidine inhibit the burst size of *E. coli* 518 phage 3 (82) and *Salmonella typhimurium* phage P-22 (6).

Probably closely related to these studies on the toxicity of spermine to microorganisms are several observations on the uptake of spermine from the suspending medium. *Staphylococcus aureus*, grown in a polyamine-free, chemically defined medium, contained only traces of spermidine and no 1,4-diaminobutane or spermine (67, 128). When the organism was suspended in buffer containing spermine, about 5 per cent of the amine was adsorbed with-

in 5 min. according to an adsorption isotherm [Razin & Rozansky (124)]. The same type of adsorption was observed with heat-killed and living organisms. It is noteworthy that more spermine was taken up by the *S. aureus* cells at pH 8 than at pH 7, and it is possible that this may explain the greater toxicity of spermine in alkaline media, as observed by Rozansky *et al.* (132) and Tabor & Rosenthal (149).

The uptake of spermine by *S. aureus* was also studied by Rosenthal & Dubin (128), who used radioactive polyamines. When *S. aureus* was grown in the presence of small amounts of C^{14} -labelled spermine, 16 to 50 per cent of the C^{14} -spermine added to the medium was taken up by the cells. On the other hand, when C^{14} -spermidine was used less than 5 per cent was found in the cells. In similar studies with *E. coli*, added spermidine or spermine (38) was also taken up by growing cells. Although, in general, the uptake studies suggested a possible correlation between uptake and toxicity, this was not absolute. For example, Razin & Rozansky (124) showed that a spermine-resistant strain of *Staphylococcus aureus* bound much less spermine than a spermine-sensitive strain. On the other hand, they showed that spermine was concentrated from the medium by the spermine-resistant *Candida albicans*, as well as by the spermine-sensitive *S. cerevisiae* (121), and by *Proteus vulgaris* and *E. coli* which are relatively spermine-resistant compared to the spermine-sensitive *S. aureus* (124).

Another aspect of the subject concerns the toxicity of spermine metabolites rather than of spermine itself. With *Mycobacterium tuberculosis* for example, Hirsch & Dubos (70, 71, 73, 74) showed that the toxic agent was not spermine but rather the oxidation product of spermine that resulted from the action of the beef serum amine oxidase. This antimycobacterial effect of spermine has been confirmed by Fletcher *et al.* (53). The same product was also toxic to *Trypanosoma equiperdum* and was more inhibitory than spermine to *S. aureus* and *E. coli* [Tabor & Rosenthal (149)]. Antimycobacterial activity was also found with the oxidation product of spermidine [Hirsch (73)].

As indicated above (pp. 586-89), the oxidation product of spermine formed by amine oxidase has not been adequately identified, although it appears to be an amino aldehyde. The synthesis of several amino aldehydes has recently been reported by Carvajal (28, 29). These compounds, β -propylal- γ -butylimine, N-(3-aminopropyl)- γ -aminobutyraldehyde, N-(4-aminobutyl)- β -aminopropionaldehyde, and an unidentified compound, were inhibitory to *Mycobacterium tuberculosis* cultures.

Since several bacteria have been shown to oxidize spermine, it is possible that comparable inhibitory oxidation products may be formed when spermine is added to bacterial cultures, thus explaining the inhibitory effect of spermine. However, inhibitory products have not yet been isolated from the bacterial oxidation of spermine, and in certain bacteria, such as *E. coli* and *S. aureus*, there is little or no evidence for the oxidation of added spermine (122, 128, 139, 151).

Chromosomal studies in plants.—Davidson & Anderson (34a) treated lateral roots of *Vicia faba* with spermine, 1,4-diaminobutane, or 1,5-diamino-

pentane and observed inhibition of chromosome-uncoiling at late anaphase and early telophase. Prophase contraction occurred, but the metaphase chromosomes were fused into a single mass. The effect of 1,4-diaminobutane had previously been studied by Marquardt (106a), who showed that this amine induced chromosome aberrations, especially translocations and fragmentations, in cells of *Oenothera* undergoing meiosis.

MISCELLANEOUS EFFECTS OF SPERMINE AND SPERMDINE

Effects of spermine and spermidine on various enzyme activities.—As already discussed in a previous section (pp. 592–95), spermidine and spermine have a stabilizing effect on the activity of prostatic acid phosphatase (79) and β -glucuronidase (15). Keister (84) demonstrated that spermine and spermidine inhibited the breakdown of RNA that occurred both with the sonically ruptured cells of various microorganisms and with crystalline RNAase (at pH 7 but not at pH 5). Similarly, spermine inhibited the DNAase action of crude sonicates and of purified DNAase upon a commercial DNA substrate. It is possible that these effects with RNAase and DNAase were not attributable to effects on the enzymes but rather to binding of the substrate nucleic acid by the polyamines; preliminary kinetic studies of Keister (84) support this concept.

Evans *et al.* (43) have reported that spermine inhibited the oxygen consumption of guinea pig brain and minced muscle in the presence of glucose, lactate, or pyruvate.

Activation of a bovine testicular hyaluronidase by spermine, spermidine, and, to a lesser extent, by several other amines has been demonstrated recently [Miyaki *et al.* (111)]. In this study, oxidation of spermine by goat serum amine oxidase destroyed its activating ability. In view of previous reports that hyaluronidase may play a role in the penetration of the egg cell by spermatozoa, these authors pointed out that the activation of hyaluronidase by spermine may have significance for fertilization.

Bichowsky-Slomnitzki (17) and Razin & Rozansky (124) studied the effect of spermine on the dehydrogenase activities of washed *E. coli* and *Staphylococcus aureus* cells toward a variety of substrates. They showed that the dehydrogenase activity of these cells toward glucose, pyruvate, and succinate was greater in the presence of spermine than in its absence. It is possible that this effect, at least in part, is attributable to the action of spermine in preventing the loss of soluble cofactors from these cells (104).

Spermidine and spermine have also been tested for their effect on two other enzyme systems with essentially negative results. Spermine, for example, had no effect on the action of prostatic vesiculase, which causes coagulation of the proteins of the seminal vesicle secretion [Gotterer & Williams (58)]. Likewise, Wright & Englert (168) have recently reported that, in contrast to protamine, spermidine and spermine do not inhibit the biosynthesis of non-saponifiable material from mevalonic acid by liver homogenates.

Agglutination of yeast, bacteria, mitochondria, and microsomes.—Spermine produced aggregation of yeast and bacterial cells [Razin, Cohen & Rozansky (121, 124)]. In the studies on bacterial agglutination, this effect of spermine

was noted in all of the Gram-positive organisms tested but in only some of the Gram-negative species; all of the Gram-negative cells agglutinated were in the rough form. The agglutination was inhibited by various divalent salts and, to a lesser extent, by monovalent salts. Agglutination was also noted when spermine or spermidine was added to suspensions of mitochondria (68, 142, 143) and microsomes (142, 143).

It seems likely that this effect of the polyamines in causing agglutination is similar to the agglutination of erythrocytes, induced by polylysine and other basic polyelectrolytes, which has been extensively studied by Katchalsky *et al.* (81).

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BIOCHEMISTRY OF CULTURED MAMMALIAN CELLS¹

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INTRODUCTION

The appearance in this series of a review dealing with mammalian cell culture reflects the broadening scope of this area of investigation in recent years. This development is in no small part the result of the simplification and improvement of techniques. The progeny of a single cell can now readily be established in culture and serially propagated for an unlimited period, and grams of material can easily be obtained for chemical and enzymatic analysis. A wide variety of cell lines can be grown either as a monolayer or suspended free in a liquid medium. These advances, among others, have permitted approaches to the biochemistry of the mammalian cell analogous to those pursued so fruitfully in the field of microorganisms. The nutritional requirements for cultured cells have been defined in chemical terms, and an important beginning has been made in the study of their metabolic and synthetic activities.

Of the extensive literature dealing with cultured cells, this review is concerned mainly with the papers published from January, 1958, to August, 1960, with reference to certain earlier papers when necessary. The indeterminate boundaries of what may properly be considered biochemistry, and thus relevant to this series of reviews, have admittedly allowed the authors considerable latitude, of which they have taken full advantage. The large volume of published material has dictated the arbitrary exclusion of certain areas. The question of the interaction of cells and viruses has been considered to be for the most part beyond the scope of this review; and the area of radiation biology has similarly not been covered, except for a few reports tangent to biochemistry. Papers dealing with cells cultivated as ascites tumors in animals but studied *in vitro* have been excluded on the technical ground that these are not strictly cultured cells. Although the distinction is admittedly artificial, particularly since some of these cells have in fact been adapted to serial propagation *in vitro* (1 to 5), the literature dealing with the biochemistry of ascites tumor cells is too extensive to be accommodated within the scope of the present article and probably justifies a separate review in which the similarities to, and differences from, serially cultured cells can be considered in detail.

As indicated below, the capacity of a cell to multiply indefinitely in cul-

¹ The survey of the literature pertaining to this review was concluded in August, 1960.

ture apparently is associated with special biochemical characteristics, so that a clear distinction should be drawn between such serially propagated cultures and either cultured tissue explants or "primary" cultures of cells freshly isolated from tissues. The latter will be considered principally from the point of view of the differences between them and established lines. The title of the review has been loosely construed to include some papers dealing with primary cultures of avian origin. Primarily technical papers have been omitted, and for these the reader is referred to recent monographs and reviews (6 to 9).

CHEMICAL CONSTITUTION

The chemical constitution of cultured mammalian cells can be defined only in approximate terms in view of the systematic fluctuations in the proportions of protein, RNA, and DNA observed during the various phases of growth (10 to 12) and of the intercellular variation in DNA content, even in the same small clone (13). Typical cells are some 80 per cent water (6), in which sizable pools of metabolites, representing about 10 per cent of the cell's total nitrogen, are dissolved. Of the pool nitrogen, 55 to 60 per cent is accounted for as amino acids and related compounds (14). There is also a considerable pool of nucleotides and other organic phosphates [(15, 16); see page 622].

The cellular macromolecules are principally protein and nucleic acid, although under certain circumstances glycogen may comprise up to 10 per cent of the dry weight (17, 18). There is approximately seven to nine times as much protein in the cell as nucleic acid; but the absolute amounts per cell may vary as much as fivefold, depending on the age and nutritional state of the culture. Logarithmically growing HeLa cells in monolayer cultures contained about 20 picograms of DNA, 46 picograms of RNA, and 440 picograms of protein per cell (11). The amino acid composition of the protein of cultured cells does not differ significantly from that of animal tissues (14). The relative amount of protein amide N in one cell line has been reported to be greater in the lag and stationary phases of culture than during logarithmic growth (19). In two cell lines, the unusual base 5-methylcytosine has been found in the DNA to the extent of 0.5 per cent (20). Under conditions in which protein synthesis and cell division are inhibited, strain L mouse fibroblasts have been reported to accumulate considerable quantities of lipid (21).

NUTRITIONAL REQUIREMENTS

The minimum essential nutrients that permit the indefinite growth of most mammalian cell lines comprise 13 amino acids, eight vitamins, salts, glucose, and 3 to 5 per cent dialyzed serum, the sole constituent not chemically defined (22, 23). The same minimal medium, supplemented only with serine, usually permits the growth of single cells (24). While supplements to this basal medium are required by certain cell lines, or by other lines under special conditions, and certain nutrients are sometimes dispensable, the close similarity between the qualitative and quantitative requirements of cells that have been derived from a wide variety of tissues and species is striking

and reflects a corresponding similarity in their metabolic capacities. Generally similar requirements have been found for cells adapted to growth in the absence of serum (25 to 30), although certain differences under these conditions were noted by Katsuta & Takaoka (31).

Amino acids.—Most cell lines have been found to require the same 13 amino acids, namely arginine, cyst(e)ine, glutamine, histidine, tyrosine, and the eight necessary to maintain nitrogen balance in man (22, 23, 32, 33, 34). A cell may possess a limited capacity to synthesize one or more of the essential amino acids, therefore rendering its presence in the medium unnecessary under certain circumstances. Thus, the glutamine requirement can be fulfilled by high concentrations of glutamic acid in the HeLa cell (35). Moreover, cells grown for many generations in glutamic acid may acquire the capacity to satisfy the glutamine requirement with low concentrations of glutamic acid (36). The ability to use glutamic acid in place of glutamine also seems to be a fairly regular characteristic of primary cultures (37, 38); in both cases the capacity is related to a relatively high level of the enzyme responsible for glutamine synthesis (39, 40). In one instance, glutamine was actually liberated into the medium by a primary culture (38). However, the utilization of glutamic acid in lieu of glutamine clearly does not change the number of amino acids required for growth.

The cystine requirement may be satisfied by providing a compound that the cell can convert to cystine, such as glutathione (23, 41, 42) or homocystine (41, 43). Under special conditions, a limited capacity to synthesize cystine *de novo* from methionine and glucose may provide amounts sufficient for growth in the absence of added cystine (43). Citrulline can replace arginine in the nutrition of many, but not all, cell lines, but no cell has been found with the capacity to use ornithine (23, 44, 45). While the conversion of phenylalanine to tyrosine has been demonstrated in a HeLa cell variant, tyrosine was not produced at a rate sufficient to alter the apparently invariable requirement for both aromatic amino acids (46, 47). The keto acid analogues can, in some cases, substitute for the corresponding amino acid (23), and dipeptides can fulfill the requirement for both constituent amino acids (48). The D-isomers of the essential amino acids are not active (49, 50).

Certain cell lines have been found to require asparagine in addition to the usual essential amino acids; this requirement can otherwise be met only with high concentrations of aspartic acid (51 to 52a). Since the steps involved in the biosynthesis of asparagine are obscure, little can be said concerning the reason for its requirement in these cells. Other usually non-essential amino acids have been shown to be necessary under certain circumstances. In certain cases at least, it is clear that the requirement is not caused by a specific deficiency of an enzyme required for synthesis of the amino acid but rather by conditions that prevent the accumulation of adequate levels in the intracellular pool. The loss of intracellular amino acids to the medium is unquestionably the explanation for the requirement for serine at low population densities (24, 43). A requirement for glycine has been shown in several instances to be related to a relative deficiency of the active form of folic acid

(37; 53 to 55), and a low level of pyridoxal has been shown to lead to a requirement for alanine (56). It is reasonable to conclude that when the intracellular level of an amino acid falls below a certain critical level, because of either reduced rate of synthesis or increased rate of loss, the cell is unable to synthesize protein and grow unless an exogenous source is provided. A similar situation may obtain in other instances in which various, usually non-essential, amino acids have been found either to increase the growth rate or to be required for growth (23, 25, 27, 31; 57 to 59a; 347).

The optimal concentrations of arginine and glutamine in the medium are generally higher than those of other essential amino acids, presumably as a consequence of the fact that they are degraded in the medium to ornithine (14, 60, 61) and glutamic acid (62), respectively, and thus made unavailable for their specific metabolic roles.

The data on the nutritional requirements for amino acids are in general borne out by studies on the utilization of the amino acids supplied in the medium (63, 64).

Vitamins, co-factors, and nucleic acid derivatives.—Cultured cells generally require choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine (65, 66); in addition, most lines also require inositol (67 to 69). In the case of a human fibroblast culture, the pyridoxal was apparently required primarily for the synthesis of the nutritionally non-essential amino acids; when these were supplied, a pyridoxal requirement could not be demonstrated (66). The effective concentrations of vitamins are very low, so that qualitative or quantitative requirements may be altered by trace contaminants contained in other constituents of the medium, particularly the serum. Coenzymes are generally able to replace their constituent vitamins, and only a few, notably folinic acid, are more effective than the parent vitamin (28, 66, 70). Primary rabbit and monkey kidney cultures apparently do not reduce folic acid efficiently, and, in these cells, 5-formyl tetrahydrofolic acid is 30 times as potent as folic acid itself (37, 54). The finding that cholesterol, ascorbic acid, coenzyme A, cocarboxylase, DNA, flavin-adenine dinucleotide, glutathione, and cysteine, in varying combination, are necessary for the growth of single HeLa cells (71) has not been confirmed (24). In the latter study, serine alone, added to a minimal medium containing only the usually essential growth factors, sufficed for the growth of single cells from a number of lines (24). The discrepancy may be referable to differences in the degree to which the cells had been traumatized prior to plating. More recently, thymidine, pyruvate, cysteine, Fe^{+++} , and hypoxanthine have been described as essential for the colonial growth of diploid Chinese hamster cells (72).

The nucleic acid bases and their derivatives are generally not required by cultured cells (22, 23, 29), although Neuman & Tytell (73) found that purines stimulated the growth of clones of the Walker carcinosarcoma cell. As mentioned above, a deficiency in reduced folic acid may result in a requirement for glycine (37). Under these conditions, there may be a requirement for thymidine and a purine as well (53, 54). The requirement for

pyridoxal and one or more non-essential amino acids may also be reciprocal (55, 66). It is clear that the requirement for a particular nutrient depends in certain instances on the composition of the medium as a whole, a fact that may explain some apparently discordant results.

Carbohydrates and salts.—A number of carbohydrates, including D-fructose, D-galactose, D-mannose, D-ribose, trehalose, and turanose, were found to substitute for glucose in supporting the growth of a variety of cell lines (74, 75). The optimal concentrations of the different carbohydrates varied widely, and differences between the responses of different cell lines were observed; e.g., strain L mouse fibroblasts were unable to grow on galactose. Generally similar, but not identical, results were obtained with a mouse lymphoblast (76) and primary chick heart cultures (77). Although glucose-1- PO_4 and glucose-6- PO_4 could be utilized in lieu of glucose (74), it is not clear whether they are transported into the cell as such or hydrolyzed by extracellular or surface enzymes. Fructose diphosphate, which was ineffective in mammalian cell cultures (74), prolonged the survival of primary chick heart explants when supplemented with ADP or ATP (78). Lactose and sucrose were incapable of supporting the growth of any of several cell lines tested (74). Although glycerol, pyruvate, lactate, and various citric acid cycle intermediates were also found to be inactive (74), Bailey *et al.* have reported a limited activity of glycerol and lactate (76). The differences may reside in the cell lines chosen for study. Variant strains have been isolated with heritable special abilities to utilize carbohydrate (79 to 81). Pyruvate, while unable itself to substitute for carbohydrate, increased the effectiveness of galactose and ribose (74).

Na^+ , K^+ , Ca^{++} , Mg^{++} , Cl^- , and $\text{PO}_4^{=}$ have been shown to be essential for the survival and growth of two lines in monolayer culture (82), but cells in suspension grow indefinitely in the absence of added Ca^{++} (83). It is not clear in the latter case whether Ca^{++} is entirely unnecessary or whether necessary small amounts are provided by the serum or other constituents of the medium. There is also a general requirement for HCO_3^- , demonstrable when the CO_2 produced by cellular metabolism is removed (84, 85). The latter requirement can be met, for reasons that are not clear, by extracts of normal cells (86). Oxalacetate also can replace HCO_3^- (87), but it may be supplying CO_2 via decarboxylation. There is a dearth of evidence on the role of trace metals, although the results of Waymouth (88) suggest that Fe^{+++} , Al^{++} , Mn^{++} , Zn^{++} , Co^{++} , and Mo^{++++} may be essential.

Proteins and other macromolecules.—Serum protein has usually been added to the medium and has long been considered essential for the serial propagation of cells. The role of the protein has recently been investigated in a number of laboratories with different, but not necessarily conflicting, results. Until recently, protein was considered to be necessary as such. Although serum albumin alone has been found to suffice (89), there are several reports that both albumin and a globulin are necessary (90 to 92). The active moiety in the globulin has been placed in the Cohn fractions IV of human serum and II and III of horse serum (93) and in the Kekwick-Mackay frac-

tion G2 (94), and it has been variously identified as fetuin (95) and as an α -globulin other than fetuin (96). Whatever the identity of the globulin, there was general agreement that it functions to promote the attachment and stretching of cells in monolayer culture and that the serum proteins also have an essential nutritional function. It should be borne in mind that some of these experiments (97) were performed at low population densities under conditions that may impose additional and special nutritional requirements.

A number of chemically undefined materials containing protein have been reported to stimulate growth of various cells. These include embryo extracts, the protein moiety of spleen nucleoprotein (98), and extracts of human tumors (99). Extracts of cells that had been adapted to a protein-free medium promoted the attachment and growth of unadapted cells (100, 101); peptones similarly have been found to be growth-stimulatory (102 to 105). Milk protein has been found to substitute for serum protein in the growth of a murine lymphoma (106); salmine has been reported to replace serum protein in an otherwise defined medium (107).

Under certain circumstances, macromolecules in the medium other than protein can play a decisive role in the survival and growth of cells. Katsuta and co-workers (108 to 112) have reported that the requirement for serum protein could be fulfilled in whole or in part by polyvinylpyrrolidone or dextran. In a human mammary carcinoma line which required an extract of umbilical cord in addition to the usual medium, highly polymerized mucopolysaccharide could replace the cord extract, and polyvinylpyrrolidone was partially effective (113). The mode of action of these macromolecular compounds is as yet unknown.

In contrast to the foregoing results, there is an increasing body of evidence that macromolecules as such are not invariably, or perhaps even regularly, required either for the growth of mammalian cells or for their attachment to glass and that the serum protein acts by supplying one or more essential growth factors of relatively low molecular weight formed or liberated on proteolysis. In the first place, a number of cell lines have been grown with yeast extract or protein hydrolysate in an otherwise defined medium (31, 100, 109, 112, 114). In some of these cases, the cells that grew in the serum-free medium were variants, in that the same medium would not support the growth of the parent strain; in other cases, the growth rate in the serum-free medium was stated to be extremely slow. There have been several reports of the serial propagation of certain cell lines in a chemically defined medium that totally lacked macromolecules (105; 115 to 118). To date, cells capable of growth in such medium have been variants that adapt to the protein-free environment, and it is not yet known how many cell lines are capable of being so adapted. The adapted variant lines do, however, attach to glass and apparently proliferate indefinitely in the absence of protein or other macromolecules. More recently, there are reports from three laboratories which indicate that most, if not all, serially propagated lines can be grown in a protein-free medium, supplemented with as yet unidentified dialysable components derived from serum protein (119 to 121). In one case (119), the

active growth factor was released from the protein by the addition of proteolytic enzymes; in the others, it was obtained from whole serum by dialysis at 37°. These observations probably are the result of the same phenomenon in view of the recent demonstration that dialyzed serum contains a complete spectrum of proteolytic enzymes capable of digesting protein all the way to the amino acid level (122).

It is clear that both the growth-promoting activity of serum protein and its action in furthering cellular adhesion to glass are often not related to the protein as such but to compounds of small molecular weight either derived from the protein, initially bound to it, or both. Although these postulated growth-active compounds can apparently be dispensed with by variants of certain cell lines, it is not clear whether their growth in a protein-free and defined medium represents a process of adaptation or the selective growth of a rare mutant.

Miscellaneous nutritional and environmental factors.—Walker carcinoma cells require pyruvate, oxalacetate, or α -ketoglutarate for growth as clones (123), and a mouse leukemia line requires either serine or pyruvate (59). Surprisingly, a concentrate obtained on heating a solution of glucose and phosphate stimulated growth of HeLa cells and promoted their attachment to glass at low concentrations of serum (124). A requirement by cells at low population densities for catalase (125) is observed only in media containing an unusually high concentration of cyst(e)ine (125a).

High O₂ tension has been found to be inhibitory or fatal to cells, the optimal concentration for growth being approximately that of air (126 to 128). D₂O at 20 per cent or more leads to cell damage and suppression of growth (129). Although the growth rate of primary rat connective tissue cells was not impaired during anaerobiosis (130), more recent results indicate a significant reduction in growth rate under these conditions in a variety of cell lines, with a concomitant increase in the rate of glycolysis (131, 132). Paradoxically, an increased glycolysis was also noted in cells growing in an atmosphere of 95 per cent O₂ (128).

There is no clear evidence that established cells require hormones, although stimulation of growth by insulin (58), somatotrophin (133), and progesterone (134) has been reported. Emulsified triglycerides or serum lipids can be utilized, although lipid ordinarily plays no necessary nutritional role (135, 136). Cells are able to metabolize serum protein (137 to 139) but ordinarily utilize it to a very limited extent as a source of amino acids for protein synthesis (140, 141).

THE METABOLISM AND BIOSYNTHETIC CAPACITIES OF CULTURED CELLS

Enzymes.—Greenstein observed that the enzyme activities of tumors are generally low and vary within narrow limits in comparison to normal tissues (142). A similar uniformity in enzyme pattern has been found in extracts of serially cultured cells, whether derived from normal or neoplastic tissue. The enzymatic activities which have been investigated include those of the citric acid cycle (143), DNA synthesis (144), hexokinase (74, 145), several

other enzymes of the glycolytic pathway (17, 146), β -galactosidase (147), transaminases (148, 149), amidases (150, 151), and others (152, 153). In general, few conspicuous differences were noted between cell lines. Although the arginase and rhodanese activities did vary significantly from strain to strain, there was no clear relationship either to the tissue of origin or to any other special characteristic of the cell (154). The presence of a given enzymatic activity in a cell extract does not necessarily indicate the role of that enzyme in the cellular economy. For example, although cells contain a variety of transaminases (148, 149), only glutamate serves importantly as an amino donor in the synthesis of the nutritionally non-essential amino acids (23).

Primary cultures generally reflect the enzyme pattern of the tissue of origin, but as the cells proliferate *in vitro* the organ-specific enzyme activities diminish until the "neutral" uniform pattern characteristic of serial cultures emerges (152, 155, 156). The reason for this phenomenon is one of the most intriguing problems in cell culture today; several alternative explanations are considered in the following pages.

There is at least one instance (36) of the control of an enzyme activity in a serially cultured cell by the product of the reaction it catalyzes. Glutamine synthase activity increased some fifteenfold when the glutamine in the growth medium was replaced by a relatively high concentration of glutamate, and it returned to its former low level when glutamine was restored to the medium. The kinetics of the appearance and disappearance of the enzyme were consistent with the interpretation that the control is at the level of synthesis of enzyme protein. The mechanism appears to be dissimilar to the inactivation of the DPNase of L cells by the substrate or its analogues (157) where the formation of a stable, inactive enzyme-substrate complex was indicated. In several instances, a specific metabolic block in a mutant cell strain has been correlated with an enzyme defect. Cultured cells from an individual with galactosemia can not utilize galactose and have been shown to have the characteristic enzyme defect of the disease, lack of galactose-1-phosphate uridyl transferase (158, 159). The development of resistance to diaminopurine and 6-mercaptopurine has been shown to be correlated with the absence, in the first case, of adenosine monophosphate pyrophosphorylase, and, in the second case, of inosine monophosphate pyrophosphorylase (160).

Amino acid metabolism.—In general, cultured cells assimilate essential amino acids from the medium and, with the few exceptions noted below, use them solely for protein synthesis (22, 23, 46, 62, 161, 162). Glutamine, however, serves not only as the precursor of the corresponding residue in protein (62) but also plays a specific role in the biosynthesis of nucleic acid bases (163, 164). Moreover, it gives rise, via glutamate, to aspartate and other non-essential amino acids (62).

There is indirect, but clear, evidence that homocysteine and cystathionine can be formed from methionine, thus providing a route for the incorporation of methionine into newly formed cyst(e)ine (23, 43). Arginine can be converted to citrulline and ornithine (14, 23, 60, 61), but the latter can not, so far as is known, be utilized by the cell as a source of arginine (23, 44, 45). The

soluble intracellular pool contains considerable amounts of taurine and glutathione (14), both presumably derived from cyst(e)ine by the usual routes. Finally, Harris & Jahnz (161, 162) have reported that part of the cell's threonine carbon is derived from serine.

Apart from the capacity to convert α -keto acids to the corresponding amino acids (23), cultured cells are able to carry out only a few reactions on the synthetic pathways of the nutritionally essential amino acids. It is interesting, but perhaps not surprising, that, with the possible exception of threonine (162), these partial reactions are limited to the five amino acids that are ordinarily essential for cultured cells but not for the whole adult animal. The capacity for glutamine synthesis has been mentioned above, but it should be noted that there is no evidence for the synthesis of glutamine from precursors more remote than glutamate and ammonia. Glucosamine-6-phosphate may possibly serve as a donor of the amide nitrogen in some instances (164). Although the capacity to synthesize glutamine to a more or less limited extent is shared by many cell lines, the hydroxylation of phenylalanine to tyrosine has been observed only in a single variant line (46). The synthesis of cyst(e)ine from glucose and methionine, presumably via serine, homocysteine, and cystathionine, has been demonstrated in a variety of cell lines but at a rate sufficient to provide little more than half the amount needed for protein synthesis in a rapidly growing cell (23, 43). Most cell lines can utilize citrulline as a precursor for arginine (23, 44, 45, 60). There has been to date no demonstration of any reaction in the pathway of histidine biosynthesis other than the utilization of the α -keto acid (23). There is no clear evidence of biosynthesis by cultured cells of any of the eight amino acids essential for nitrogen balance in man, apart from the amination of the keto acid analogues. Whether there is a specific metabolic block or whether the entire pathway is lacking remains to be determined.

The evidence available concerning the biosynthesis of the non-essential amino acids is in accord with the pathways previously shown with other systems, and few new insights have been obtained. When cells are grown on a minimal medium, the non-essential amino acids fall into two groups, those derived mainly from glucose (alanine, serine, and glycine) and those derived mainly from glutamine via glutamate (asparagine, aspartate, and proline) (23). The α -amino group of the non-essential amino acids is also derived primarily from glutamine via glutamate; ammonia and the amino groups of other amino acids are utilized to a negligible degree (23, 62, 65).

The glucose family of amino acids (serine, glycine, and alanine) must be further subdivided into two groups. There is only negligible interconversion between alanine and either serine or glycine. Further, when cells are grown in a medium containing ribose and pyruvate instead of glucose, alanine derives almost wholly from pyruvate, whereas serine and glycine derive from ribose (165). In the normal cell, glycine and serine have a common source and are interconvertible (23). However, the conversion of glycine to serine proceeds much less freely than that of serine to glycine, and provision of exogenous serine in a medium containing glucose- C^{14} dilutes the C^{14} label in glycine

and serine to precisely the same degree (165). In folic or folinic acid deficiency exogenous glycine must be provided (37, 53, 54, 55). These observations indicate that serine is the direct precursor of glycine, rather than vice versa.

The carbon chains of aspartate and asparagine are derived from glutamate via unknown intermediates (23, 62). It is of interest that a degree of vitamin-B₆ deficiency, which considerably impaired the synthesis of other non-essential amino acids, had no demonstrable effect on aspartate synthesis (166). There is an alternative route to aspartate from glucose which is of some importance in HeLa cells (23). The amide nitrogen of asparagine is derived from the amide nitrogen of glutamine by a pathway that apparently does not involve free ammonia (167). Beyond this fact, the biosynthesis of asparagine is as obscure in cultured cells as it is elsewhere. Proline apparently is formed from glutamate by way of glutamic semialdehyde, but this pathway accounts for only approximately half of the proline (23). The origin of the remaining proline is not known; ornithine has apparently not been investigated as a possible precursor. When cells grow in a medium initially lacking the non-essential amino acids, variable but significant amounts are liberated into the medium, reflecting the equilibration of the latter with the cellular pool.

Protein metabolism.—A number of early reports had suggested that the proteins in the medium are used to an important degree for the synthesis of cell protein. However, more recent studies indicate that, although under certain circumstances there may be extensive degradation of the medium protein (137 to 139; 164, 168), neither serum proteins nor the proteins in cellular extracts are used to a significant degree for the synthesis of cell protein (140, 141). The latter observation is in accord with the increasing body of evidence that, although protein in the medium may facilitate adhesion to glass by an as yet undefined mechanism, its nutritional role consists in the provision of as yet unidentified small molecules other than amino acids.

The precursors for the synthesis of cell protein are amino acids in the intracellular pool. When the intracellular concentration of a given amino acid falls below a critical threshold level of about 0.01 mM, net synthesis of protein is halted (39). There is relatively little information presently available on the sites of protein synthesis or the interrelations of protein and nucleic acid synthesis in cultured cells. Harris (169) has determined by radioautography that amino acids are incorporated in the nucleus and cytoplasm at roughly the same rate; the latter site accounted for four-fifths of the total uptake in one experiment. When the synthesis of both nuclear and cytoplasmic RNA was sharply curtailed by metabolic antagonists, there was virtually no inhibition of the incorporation of amino acids into protein, indicating no necessary relationship between the over-all synthesis of RNA and protein (169). In irradiated HeLa cells, the incorporation of amino acids was found to continue after DNA synthesis had been halted (170).

A number of metabolic antagonists of amino acids have been reported to inhibit growth of cultured cells, presumably by inhibiting protein synthesis; these include *p*-fluorophenylalanine, β -thienylalanine, fluorotyrosine,

ethionine, and canavanine (47; 171 to 173). Canavanine has been found to be incorporated to an appreciable degree into the protein of Walker carcinoma cells, apparently at the expense of arginine (174). A paradoxical effect has been obtained in one instance with β -thienylalanine: RNA synthesis was suppressed with no appreciable effect on protein synthesis (169).

There are several reports concerning the synthesis of immunologically or chemically identifiable proteins in cultured cells; these will be discussed in the following paragraphs. The adaptive formation of the enzyme glutamine synthase has already been mentioned.

Carbohydrate metabolism.—In a limiting medium containing only demonstrably essential growth factors, cultured mammalian cells derive energy largely, if not entirely, through the utilization of carbohydrate. Although they are generally capable of respiration and can oxidize carbohydrates to CO_2 (84, 85, 159, 175, 176), the glycolytic pathway generally predominates when glucose is the substrate (74; 177 to 180). This relatively high rate of glycolysis leads to the accumulation of lactic acid in the medium and of smaller amounts of pyruvate, oxalacetate, and α -ketoglutarate (74, 181, 182). The quantity of carbohydrate utilized and the degree of aerobic glycolysis bear no necessary relationship to the growth rate but are profoundly influenced by such diverse factors as the nature and concentration of substrate (74), the age and nutritional state of the culture (175; 183 to 186), the pH and O_2 tension in the medium (128, 131, 132, 187), the cobalt concentration in the medium (188), or even the nature and concentration of the serum supplement (17, 189, 190). In view of these considerations, one can only state that cultured cells generally exhibit the relatively high aerobic glycolysis described by Warburg as characteristic of cancer tissue, but that cells originally deriving from normal and malignant tissues do not differ in this respect (74, 175, 177, 179, 191). This, however, does not necessarily imply that normal cells regularly become malignant in serial culture.

The utilization of other sugars in lieu of glucose has been discussed in the section on nutrition. The rate of glycolysis with different cell lines appeared to be determined in large part by the rate at which the particular substrate was phosphorylated (74). On the other hand, when one of these cell strains was studied as a thick suspension in a Warburg vessel with glucose as a substrate, the factor limiting glycolysis was its supply of endogenous substrate, inorganic phosphate, or adenine nucleotides (17). The inability of a mutant cell to utilize galactose has been correlated with a specific enzyme defect (158, 159).

The glucose analogue 2-deoxyglucose has been found to inhibit glycolysis and growth in a variety of cells. It is phosphorylated by hexokinase in these cells, and the phosphorylated compound inhibits several steps in the glycolytic pathway (192), particularly glucose-6-phosphate dehydrogenase and phosphohexose isomerase (193). Insulin (194, 195), hydrocortisone (196), and thyroxine (197, 198) have been reported to increase aerobic glycolysis. The effect of insulin is observed only in certain cell lines; but the finding that it is effective only in cells originating from malignant tissue (194) has not

been confirmed. Dinitrophenol at concentrations of 10^{-4} M increases respiration of intact cells, presumably by uncoupling oxidative phosphorylation (176, 199), whereas antimycin, azide, malonate, and fluoropyruvate inhibit respiration (176, 200).

Apart from its role in energy metabolism, glucose serves as the precursor of a number of important cell constituents. In addition to the nutritionally non-essential amino acids and the compounds derived from them, glucose is the source of the nucleic acid ribose and deoxyribose. The results of experiments with isotopically labeled glucose are consistent with the view that ribose is formed from it by both the oxidative and non-oxidative pathways and that the deoxyribose is, in turn, formed from the ribose. Some evidence has also been obtained suggesting a possible role of triose phosphate in deoxyribose synthesis (201, 202). Appreciable amounts of glycogen have been found in some cells (17, 18) and none in others (201). The variation appears to depend both on the cell line and the conditions under which it is grown. Although exogenous inositol is required for the growth of almost all cultured mammalian cells (67), significant amounts are synthesized from glucose (68). Inositol-independent variants of some human lines have been described (69). An inositol-independent mouse fibroblast which synthesized all of its inositol from glucose released into the medium an amount sufficient to feed inositol-dependent strains grown parabiotically with it (68).

Nucleic acid metabolism.—As mentioned above, the sugar moieties of nucleic acid in cultured mammalian cells arise from glucose. The cells can also synthesize the nucleic acid bases *de novo* from constituents of the minimal medium by biosynthetic pathways generally similar to those in other mammalian systems. In HeLa cells, as in other mammalian systems, the amide N of glutamine is the direct precursor of 2 N atoms of the purine ring, and of the guanine amino group. Additionally, the glutamine amide N, rather than ammonia, is the precursor of one pyrimidine ring N and of the cytosine amino group. The remainder of the pyrimidine ring appears to be formed in the usual manner from aspartate (163). Exogenous formate and glycine are also incorporated into nucleic acid, presumably into purines and thymine (203 to 205). 5-Amino-4-imidazole carboxamide can also be used for purine synthesis by surviving bone marrow cells (206). The formation of DNA thymine is folic acid-dependent (53, 54), presumably at the methylation of deoxyuridylate. The enzymatic synthesis of DNA has been demonstrated in HeLa cell extracts (144).

When preformed bases are provided in the medium, they are generally preferentially utilized (15, 204, 205). The degree to which a particular exogenous base or a derivative compound is used as a nucleic acid precursor depends on many factors, including its rate of entry into the cell, its rate of degradation in the medium, the size of preformed intracellular pools, and its possible effect on the pathways normally operative in *de novo* synthesis.

Cell cultures are well-adapted for radioautography, and this technique has been used in the investigation of such questions as the site of RNA synthesis. In these experiments the cultures were usually exposed to a radio-

actively labeled precursor for a short period, and the conclusions are subject to the general reservation that an atypical small fraction of the nucleic acid may thereby be labeled. Subject to this caveat, the chromosomal portion of the nucleus appears to be the primary site of nuclear RNA synthesis (207, 208), and the nuclear RNA then migrates to the cytoplasm as a macromolecule (209, 209a). Harris (169, 209b), however, has pointed out that the rapid turnover of RNA complicates the interpretation of these data, and he has presented evidence for the independent synthesis of nuclear and cytoplasmic RNA.

Radioautography has also been applied to investigation of the time relationship between DNA synthesis and mitosis. In HeLa (210) and L cells (211), a postmitotic resting phase of some eight to 12 hours was followed by an equal period of DNA synthesis and then by a shorter premitotic resting phase. The division phase itself required less than an hour. Similar results have been obtained by the chemical analysis of cell cultures partially synchronized by treatment with amethopterin or fluorodeoxyuridine (212). In cultures partially synchronized by chilling, however, evidence has been obtained of an additional period of DNA synthesis in the early postmitotic phase (213, 214).

With respect to the relationship between RNA and DNA synthesis, the uptake of precursors into the RNA of non-dividing cells has been found to continue in the absence of DNA synthesis or turnover (215); in primary rat heart explants, however, DNA synthesis required prior or concomitant RNA and protein synthesis (173). RNA synthesis has been found to stop during cell division (208, 216).

Additional evidence on the nucleic acid metabolism of cultured cells has been obtained with metabolic antagonists. 5-Fluorodeoxyuridine and 5-fluorodeoxycytidine inhibit DNA synthesis and cell growth at concentrations of about 10^{-7} M (217 to 219). The inhibition is prevented when equimolar thymidine is provided with the analogue. As in other systems (220, 221), these compounds therefore appear to interfere with the synthesis of the thymine moiety of DNA. After cells have been exposed to fluorodeoxyuridine for 24 hours, thymidine no longer reverses the toxicity; in this case unbalanced growth has apparently led to cell death (217, 218). The ribosides of the fluorinated pyrimidines are also toxic to cells, but a 100-fold molar excess of uridine or cytidine reverses the toxicity, whereas thymidine does not (217, 219, 222). It appears likely that, as in other mammalian systems, there is interconversion of the ribosides and deoxyribosides to some extent and that, with either type of precursor, fluorinated pyrimidine moieties are incorporated into RNA. The fluorinated pyrimidines themselves are also effective inhibitors, probably to the extent that they are converted to ribotides or deoxyribotides (221, 223).

Bromo- and iododeoxyuridine, unlike the fluoro analogue, are incorporated into DNA, replacing up to 50 per cent of the thymine. Cells containing such "false" DNA are ordinarily capable of no more than one or two divisions (20; 224 to 227); but Djordjevic & Szybalski (228) have described a

cell line that is capable of serial growth in the presence of bromodeoxyuridine, with about one-half of the thymidine residues replaced by bromouracil, which is present in both DNA strands. The DNA of these cells is reported to replicate in the semiconservative manner predicted by the Watson-Crick model (228). 6-Azauridine was not incorporated into the nucleic acid of sarcoma-180 cells but inhibited growth by interfering with pyrimidine synthesis (229).

6-Mercaptopurine is a potent inhibitor of cultured cells, apparently by interfering with purine synthesis (230 to 232), and is reversed by hypoxanthine or adenine (230). Unsubstituted purine acts in a similar manner, although it is not as active as mercaptopurine (230). 8-Azaguanine inhibits growth by suppressing RNA synthesis, by mechanisms that are not clear (169, 233, 234), and 4-aminopyrazolo-3,4-*d*-pyrimidine may also exert its cytotoxic effect by inhibiting RNA synthesis (235).

The responses of different cell lines to the foregoing groups of compounds are somewhat variable; in at least several instances, this has been shown to be related to such factors as their varying ability to take up and metabolize either the antimetabolite or the compound capable of reversing its action (229, 236). The isolation of cell variants resistant to some of these compounds is discussed in a following section.

Some effects of folic acid antagonists have already been mentioned in the section on amino acid metabolism. In the presence of amethopterin or aminopterin, cells require exogenous glycine, thymidine, and a purine source for survival and growth (53, 237). Under these circumstances, thymidylate, 5-methyldeoxycytidine, and, at least temporarily, bromodeoxyuridine can substitute for the thymidine (53, 224); the requirement for purine is generally filled by hypoxanthine, adenine, their nucleosides, or nucleotides (53). Evidence has been presented that nitrogen and sulfur mustards act by inhibiting nucleic acid biosynthesis at a point beyond the mononucleotide stage; in L cells, DNA was affected to a greater degree than RNA (238).

The toxic effect in L cells of adenine at concentrations above 10^{-3} M can be reversed by either uridine or cytidine, suggesting that the adenine may act by depleting the cell of phosphoribosylpyrophosphate required for the synthesis of pyrimidine ribosides (239).

Other aspects of cell metabolism: miscellaneous growth inhibitors.—Several cultured cell lines have been shown to carry out transformations of the steroid hormones, a function not previously considered a general property of mammalian tissues. Although different lines had somewhat different capacities in this respect, in no case were non-steroid products formed (240 to 243). A number of cultured cells have been found to produce a plasminogen activator (244).

A wide variety of substances other than those mentioned in preceding sections has been found to inhibit the growth of cultured cells, the effective concentrations varying from 10^{-4} to less than 10^{-9} gm/ml. Approximately 80 per cent of the compounds active against tumors *in vivo* were cytotoxic

in vitro at concentrations of 10^{-4} gm/ml or less. In most instances, the mechanism of toxicity was not apparent (245). Conversely, the more actively cytotoxic a compound on cultured cells *in vitro*, the greater was the probability that it would have an antitumor activity *in vivo* (245). The effects of hydrocortisone, insulin, and thyroxine on carbohydrate metabolism have been described in a preceding section. In addition, hydrocortisone has been reported to have cytotoxic effects at 10^{-6} gm/ml (246) and to partly inhibit cell growth at around 10^{-4} gm/ml (247, 248). Estrogens have been reported to inhibit the growth of HeLa cells at 2×10^{-6} gm/ml (249). Thyroxine was inhibitory for primary fetal lung cultures at 10^{-4} M but not for a number of other cell lines (198).

Antibiotics differ widely in their inhibitory effect, from penicillin and streptomycin, which are non-toxic at concentrations as high as 10^{-2} – 10^{-4} gm/ml (8, 250), to actinomycin, which causes a 50 per cent inhibition of growth at concentrations of approximately 10^{-9} gm/ml (245, 251, 252). Carbon monoxide inhibits cell growth, possibly through an effect on cytochrome oxidase (253). The toxic effect of high O_2 tension has already been discussed. A crude pancreatic extract ("trypsin") inhibited growth at concentrations in excess of 1 mg/ml (254), as did crystalline trypsin (255). Trypsin-damaged cells were found to require 10 times as much serum in order to permit their adhesion to glass (94, 255), and the toxic effect of crystalline trypsin was reversible with trypsin inhibitor (255). The inhibitory effect of liver extract (256) has been shown to be attributable to its arginase content (257); the enzyme destroys the arginine in the medium, and this leads to a nutritional deficiency.

Diphtheria toxin has also been found to be cytotoxic for a number of cell lines (258 to 260), and variants resistant to its action have been isolated (258). The earliest metabolic change consisted of a decreased incorporation of inorganic phosphate into ATP, followed by a decreased synthesis of protein and RNA. Cells deriving from a toxin-resistant animal (mouse) were also resistant to the cytotoxic effects in culture (260). An undefined toxic substance not present in normal serum has been found in the serum of burned children (261); but individual sera from apparently normal donors, both human and animal, may also fail to support growth. It is not clear whether such sera lack a necessary growth factor or contain an active inhibitor. Bjorklund (262) has described the presence in normal human serum of an active cytolytic factor normally bound to an inhibitor, but dissociable from it. The continued presence of a clumping factor for human cells in repeated serum specimens from the same donor has also been described (263). The absorption of calf serum with human or rabbit erythrocytes has been found to improve its growth-promoting activity for human and rabbit cells, respectively (264). It is of interest to note that serum from patients with Hashimoto's disease was found to be specifically cytotoxic for thyroid cells in primary culture. The cytotoxic agent was inactivated at 56° and was not then reactivated by the addition of complement (265).

TURNOVER OF MACROMOLECULES

When cultured mammalian cells are placed in a medium containing a C^{14} -labeled amino acid but deficient in one or more other essential amino acids, there is, of course, no net synthesis of protein, but labeled amino acids are incorporated into protein at the rate of 0.75 to 1 per cent per hour (46; 266 to 268). This process of protein renewal involves a major portion of the cell protein, for it can continue at the same rate for as long as 72 hours, by which time more than half of the amino acid residues have been replaced. A similar result in reverse is obtained when prelabeled cells are placed in a medium containing the corresponding unlabeled amino acid. There is as yet no information as to whether this average rate of turnover, common to all the cells so far examined, masks important differences in the turnover rate of individual proteins. The data of King *et al.* (269) do indeed suggest that a fraction of the cell protein may be turning over much more slowly than the rest. In resting yeast (270) and bacteria (271), the rate of protein turnover is much greater than it is under conditions of rapid growth; in mammalian cells, however, it appears to be the same whether the cell is growing or resting. Relative to the growth rate of 3 per cent per hour, the 1 per cent rate of protein turnover in mammalian cells is much greater than in either bacteria or yeast. Surprisingly, it is unaffected by the omission of glucose, although it is halved by the omission of glutamine (266). The turnover process is generally believed to be one with balanced degradation and resynthesis. However, the possibility that the amino acid residues of a protein molecule can be replaced by an exchange mechanism which does not involve total degradation to the amino acid level has not been rigorously excluded.

The situation with respect to the turnover of nucleic acids is far from clear, in part because of the difficulty of identifying the molecular level to which the postulated degradation proceeds prior to resynthesis and, thus, the level at which it could be detected isotopically by the use of the appropriate metabolic trap [cf. (271)]. This is illustrated by the finding that, after inorganic P^{32} had been incorporated into nucleic acid by mouse fibroblasts (strain L), the label was conserved after five generations of growth in an unlabeled medium (272). This result does not, however, prove stability of the nucleic acid, for the turnover of the nucleic acids might involve their degradation to, for instance, polynucleotides or nucleotides which do not equilibrate with the inorganic phosphorus of the medium and pool. In contrast, when cells were labeled with C^{14} -formate and then grown in a medium containing unlabeled formate, there was progressive loss of label from cytoplasmic and nuclear RNA; no loss of label from DNA was observed under these conditions. When thymidine was added to the medium, there was a limited loss of label from DNA, suggesting the possibility of a slow DNA turnover which is ordinarily masked by recapture (204). In non-growing rabbit macrophages, labeled adenosine was incorporated into RNA at a rate of approximately 2 per cent per hour, whereas DNA was not labeled, indicating a stability of the DNA in this non-growing system (215). In HeLa cells which had been labeled by exposure to cytidine- H^3 for 90 to 120 min.,

there was more rapid turnover of soluble RNA than of RNA of higher molecular weight (216). It is apparent from the foregoing that the apparent turnover of nucleic acid is dependent to a large measure on the mode of labeling, the nature of the trapping agent, and the efficiency of recapture of the breakdown products; moreover, the last factor in turn is influenced by the growth rate of the culture (204). The only general statement possible at this juncture is that, while RNA turnover occurs under most conditions of mammalian cell culture, DNA is considerably more stable.

TRANSPORT AND METABOLIC POOLS

Although cultured mammalian cells, particularly when grown in suspension, would appear to lend themselves admirably to kinetic studies on the uptake of small molecules, they have been used for this purpose to only a minor degree. The extensive studies of Christensen, Heinz, and others [cf. (273)] on amino acid transport by surviving ascites cells, and similar studies with respect to glucose and phosphate transport, have not as yet been duplicated with serially propagated cells in the logarithmic phase of growth. The limited information available indicates a rapid concentrative uptake of most of the nutritionally essential amino acids, reaching approximate equilibrium within 30 to 60 min. At physiologic concentrations, most of the amino acids are concentrated two- to fivefold (14), although a few, notably threonine, can be concentrated to a much greater extent (39, 43).

In addition to the compounds taken up from the medium, the cellular pool contains significant amounts of the nutritionally non-essential amino acids synthesized from glucose and glutamine; their absolute levels in the HeLa cell pool vary from 1.4 mM in the case of alanine to (approximately) 0.03 mM in the case of serine (14). In addition, HeLa cell pools were found to contain, in decreasing order of concentration, taurine (14 mM), glutathione (4 mM) glycerophosphoethanolamine (1.4 mM), β -alanine and urea (0.5 mM), γ -aminobutyric acid and ornithine (0.1 mM), and a number of additional as yet unidentified, ninhydrin-reactive compounds. Ammonia is present almost entirely as a hydrolysis product of glutamine. Most of these compounds synthesized by the cell are retained concentratively; the ratio of intracellular:extracellular levels varies from approximately 7000 in the case of taurine to 10 to 30 for the non-essential amino acids, two for ornithine and ammonia, and one for urea (14).

In view of the enormous quantitative disparity between the volume of cells and medium, whether in suspension or monolayer culture, even a high degree of retention by the cell involves the loss to the medium of significant amounts in absolute terms. (A cell count of 250,000 cells per ml. culture fluid represents a volume ratio of approximately 1:1000.) At relatively low cell populations, the loss of serine to the medium exceeds the biosynthetic capacity of cells, and they do not then grow unless exogenous serine is added to preserve the cellular pool (24). Similar results have been obtained in the case of cells placed in a cystine-free medium; they survive and continue to synthesize cystine only at extremely high population densities. At lower cell

counts, exogenous cystine must be added in order to conserve the intracellular cystine pool and permit cellular survival (43). There is no direct evidence of the role of pyridoxal phosphate in amino acid transport in these cells; cells deficient in vitamin B₆ to a degree that interfered with amino acid synthesis still possessed a normal capacity to take up and concentrate amino acids from the medium (166).

Relatively little is known concerning the transport of carbohydrates into the cell. Agol (274) found no evidence that either glucose or galactose was concentrated to any extent by several cell lines, a finding that has been confirmed for glucose (275). On the other hand, Maio & Rickenberg (276) have recently reported that galactose and glucose are concentrated, the former as much as thirtyfold, by L cells. The two sugars appeared to have a common transport mechanism, and the concentrative uptake was inhibited by dinitrophenol and iodoacetate. In the latter study, however, no clear distinction was drawn between the hexose and its metabolic derivatives within the cell. All four of the mesoinositol monophosphate isomers proved as active in supporting growth as inositol itself (165). It is not yet clear whether these are transported into the cell as such or are hydrolyzed extracellularly. No information is available as to the concentrative uptake of vitamins or co-factors.

Nucleic acid bases, nucleosides, and nucleotides can enter cultured cells, but the form or forms that actually traverse the cell membrane during absorption are not known. In the cellular pool these compounds are largely, if not exclusively, in the nucleotide form. Adenine nucleotides, principally ADP and ATP, constitute approximately half the soluble nucleotides of HeLa cells, and the acid-soluble adenine amounts to approximately one-quarter of the total adenine of the cell. Uracil derivatives are second in order of abundance, with considerably smaller amounts of cytosine and guanine nucleotides and no detectable thymidylic acid (15, 16).

The total intracellular soluble phosphorus amounts to about 35 μ M per ml of cell water; of this soluble phosphorus the nucleotides account for about three-fifths. The remainder includes sugar phosphates, glycerophosphoethanolamine, and inorganic phosphate. There is no evidence for the concentrative uptake of the latter (16, 275). Repeated washing in isotonic saline solution leads to increased cell permeability and loss of intracellular phosphorus (277).

Polymerized homologous DNA has been shown to be taken up as such by a cultured cell (278, 279); this at least suggests the possibility of transformation experiments with mammalian cells.

SPECIALIZED FUNCTIONS IN CELL CULTURES

Most of the biochemical studies with mammalian cell cultures have dealt with the nutritional requirements and metabolic processes associated with cellular growth. For the most part, these studies underscore the essential biochemical unity of growth processes in living cells. There have been differences between individual mammalian cell lines; but, in general, all the mam-

malian cell cultures so far studied, whatever the species of origin, whether deriving from adult or embryonic tissue, fibroblastic or epithelial, normal or malignant, have proved to have essentially the same nutritional requirements and to use those nutrients by the same pathways to effect the synthesis of essential metabolites and macromolecules.

It should be emphasized, however, that this work relates in large part to the metabolic processes associated with cellular growth. With only a few exceptions, to be discussed, the highly diversified functions of differentiated cells in the intact animal have not been reproduced in serially propagated cultures. Most of the reports of sustained function in tissue cultures deal with primary cultures of organized tissue and are therefore not directly relevant to the problem under discussion. The following, and many similar, studies describe the continuation of specialized functions for periods of up to 400 days in surviving bits of tissue: the production of collagen in explants of chick lung tissue (280, 281) or of guinea pig granulomata (282); the continued production of hyaluronic acid or chondroitin sulfate by explants of avian embryonic brain, fetal skin, and bone (283) or by human synovial membrane (284, 285); the production of insulin by chick embryo pancreas (286); of milk proteins by mammary gland cultures (287); of catecholamines by explants of adrenal tumors (288); of gonadotrophin by a hydatid mole (289); and the production of antibodies by splenic fragments (290). Although there was often peripheral cellular growth around many of these explants, there was no basis for ascribing the persistent function to the proliferating cells, as distinct from the organized and surviving explant, or to cells that had wandered out from it. Significantly, in explant cultures of human melanomata, although pigment-containing cells were observed in the peripheral outgrowth, these were never seen in mitosis, whereas the cells that grew out of trypsinized tissue were not pigmented. The implication is clear that the pigmented cells in the primary culture were surviving melanin-producing cells which had merely migrated out of the original explant (291).

The foregoing reports are relevant to the problem of continued function in dispersed cell cultures only to the degree that such explant cultures afford an experimental approach to the conditions and metabolites necessary for the continuation *in vitro* of highly specialized metabolic activities. More directly pertinent to the problem under discussion is the fact that when tissues are dissociated into their component cells, the latter in primary culture may continue to carry out a metabolic activity characteristic of the organ of origin. Thus, dissociated chick (292 to 294) and rat (295) muscle or heart cells in primary culture have continued to pulsate for many days; however, this property was lost on subculture. Hydroxyproline was formed in primary cultures of rabbit blood (296), chick embryo heart (297), and dispersed chick osteoblasts (298, 299). Porter & Pappas (300) have adduced electron-micrographic evidence that in primary cultures of chick embryo dermis fibroblasts, unit fibrils of collagen are formed in close association with the cell surface. Cells from human granulocytic and monocytic leukemia differentiated in short-term culture to peroxidase-positive polymorphonuclear forms and

macrophages, respectively (301). Cultured bone marrow cells retained for 10 days their capacity to produce recovery in mice that had received an otherwise lethal dose of radiation (302). Finally, it has been shown in a number of laboratories (303 to 308) that cells isolated from the lymph nodes of animals previously immunized with a specific antigen continued to form antibody *in vitro*. There is a recent report also that cells deriving from animals that had not been previously conditioned to a specific antigen could be induced to form antibody *in vitro* by a primary exposure to that antigen. When either phage T2 or hemocyanin was incubated at 37° with packed rat cells from a peritoneal exudate and the mixture then extracted, the modified antigenic material in the extract induced an antibody response in previously unconditioned cells deriving from a rat lymph node (309). A similar primary antibody production by cultures of rabbit monocytes has been described by McKenna & Stevens (310).

The foregoing reports deal with freshly isolated cells in primary culture which are either not multiplying or have gone through, at most, a few divisions. Such functioning cells can either not be serially propagated or, if propagated, generally lose their capacity to function. It is nevertheless significant that dispersed cells can, under certain conditions, continue to carry out functions normally carried out by an organized intact tissue and that cellular dispersal as such does not necessarily lead to the loss of the capacity of the cell to carry out specialized biochemical activities. In the instances cited, that function was apparently related to a single cellular type, and neither tissue organization nor continuing contributions from other cellular types appeared to be essential for the continuation of function.

There have been a few reports of continued specialized metabolic functions in serially propagated cell cultures. Neoplastic mast cells in culture continued to synthesize histamine (311, 312) and 5-hydroxytryptamine (312, 313). However, although a culture of mast cells studied by Williams *et al.* (314) retained the characteristic basophilic granules, it did not elaborate these amines. Cultures of anterior pituitary gland have been reported to elaborate growth hormone (315). Serially propagated mouse fibroblasts in suspension culture have been reported to form collagen fibers (316 to 318); but the identification of the fibers as collagen was based on electron microscopy or enzymatic susceptibility, and direct chemical confirmation has not yet been obtained. Labeled amino acids have been reported to be incorporated by chick fibroblast and HeLa cell cultures into homologous serum albumin (319). Although certain mouse liver cultures have been reported to contain 25 times as much glycogen as a mouse fibroblast (320), in subsequent studies some of the liver lines had no demonstrable glycogen, whereas a non-liver strain was glycogen-positive (18).

The persistence of specific hormonal susceptibility is obviously relevant to the problem of the maintenance of function. Although the reported inhibition of the growth of a uterine cancer cell by sex hormones (249) is of interest in this connection, there was no information on the strain specificity of the response. Progesterone and testosterone were converted to a variety of

steroids, not only by uterine fibroblasts, but by suspension cultures of fibroblasts originating from mouse subcutaneous tissue as well (242). This may therefore be a general metabolic activity of growing cells rather than a specialized function limited to, for example, uterine cells. This probably applies also to the synthesis of cholesterol by human uterine fibroblasts (321) and of lipids by human leucocytes (322).

Except for the instances cited above, most serially propagated cultures do not generally carry out the specific biochemical activities characteristic of the organ of origin (2, 152, 156; 323 to 326). Two general explanations have been considered: "dedifferentiation," and failure of the specific functioning cell to grow in culture.

"*Dedifferentiation.*"—One possibility is that the specific functioning cell of an organ loses that function when grown in culture. It must be emphasized that, although the term "dedifferentiation" has been applied to this phenomenon, there is no evidence that it represents a reversion to the embryonic, multipotential, and as yet undifferentiated state [cf. (327, 327a)]. Whatever the mechanisms involved, the enzymatic activities necessary for the specific cells functions are often absent or greatly decreased in serially propagated cell (cf. 324, 325), sometimes within a few days after the original tissue had been placed in culture (152, 155, 156; 328 to 330). This could reflect the loss of enzymes because of the cellular manipulation and trauma incident to the original culture. It could also, however, be part of the general decrease in enzymatic activity frequently observed within the first few days of cellular culture *in vitro* and not limited to the functional enzymes characteristic of the organ of origin (152, 156, 329). Alternatively, the specific enzymes necessary for differentiated function, or the machinery for their production, could be present but inactive, e.g., for lack of appropriate precursors or cofactors. The effect of acetylcholine in preserving the cholinesterase activity of cultured chick embryo intestine (330) is pertinent in this connection.

The thesis that functioning cells rapidly lose their capacity to function in culture is strongly supported by the similar rapid loss of organ specificity recently reported by Weiler (331). In primary cultures of hamster kidney, organelle-specific cell types (glomerulus and tubule) could be distinguished by their varying susceptibility to specific antisera and by radioautography with I^{131} -labeled antisera. Identifiable clones from such cells retained their antigenic specificity for only three to four days and thereafter could no longer be distinguished immunologically. Selective overgrowth was not considered a tenable explanation for the observed changes.

It is possible also that specialized function may require biochemical interaction between a number of cell types in the same organ or the provision of humoral factors which are not available in the usual cell culture media (332, 333). Further, the cellular population density may be critical to function [cf. (334)], and the spatial orientation of the cells may also be important. Trinkaus & Groves (333) and Grobstein (335) have discussed the importance of cellular interaction and structural organization in morphogenesis and embryonic differentiation. It is of interest in this connection that embryonic chick cells

dissociated by trypsin reaggregate in culture to form tissue-like groupings resembling those of the tissue of origin (336 to 340); in the case of pre-cartilage cells, these groupings proceeded to lay down a typical cartilage matrix (339). Further, cultured monkey kidney cells implanted into rat brain arranged themselves into tubular patterns characteristic of the parent organ (341). It has been suggested also that the enzymes necessary for function may be reproduced more slowly in a rapidly growing cell than is the rest of the cell substance. An old observation of Doljanski (342) is illustrative in this connection; when chick iris epithelium was cultured, pigment disappeared, but when cellular multiplication was then slowed by appropriate modification in the medium, pigment reappeared in the cultured cells.

Failure of the functioning cell to grow in culture.—Instead of a loss of function by ill-defined and perhaps multiple processes of "dedifferentiation," it may well be that the cells that grow out in culture are not those directly concerned with a specific metabolic activity. It is of interest in this connection that both fibroblastic (343) and epithelial (344) cells have been grown from the buffy coat of centrifuged blood and bone marrow. In such cultures, Woodliff has been able to follow the progressive overgrowth of the normal differentiated leucocytes, first by granular macrophages and later by fibroblasts (345). More significant, fibroblastic cells growing out of chick embryonic cartilage failed to lay down matrix, even after implantation into the chick allantoic membrane (346), and young cultures of liver (155) or kidney (152, 328) cells lacked enzymes characteristic of the organ of origin. As indicated in the preceding section, these observations do not exclude the possibility that the cells which had grown out were indeed derived from the functioning cells but had very rapidly lost their capacity to function. More recently, however, Sato, Zaroff & Mills (155) have shown that the first cells to migrate out of a liver explant not only lacked the specific enzymatic activities characteristic of liver tissue but did not react with an antiserum specifically cytotoxic for parenchymatous cells. The latter observations, coupled with the fact that less than 0.1 per cent of the liver cells actually grew out in subculture, strongly suggest that in this instance there had been a selective multiplication of non-parenchymatous cells.

The elucidation of the factors responsible for the maintenance of function in dispersed cell cultures is of obvious major importance. The fact that, in a number of reasonably well-documented instances, dispersed cells in primary culture and even serially propagated and rapidly growing cells may continue to carry out specialized function offers hope that this may be a fruitful area for future investigation. However, until the conditions necessary for the maintenance of function in dispersed cell culture have been clearly established, such cultures can hardly be used to explore the mechanisms underlying cellular differentiation and embryonic morphogenesis.

CELLULAR VARIATION AND MUTATION

Although there is a basic similarity in the nutritional requirements and metabolic activity of most mammalian cell strains so far studied, a number

of minor differences have been noted. Some of these differences have been evident on the very first attempt to culture the cell strain *in vitro* (23, 51, 55, 57, 59, 59a, 347). A second type of variation often appears in the course of serial propagation and is evidenced by the diverse cell types that can be isolated from a single cell strain, diverse with respect to morphology, karyotype, nutritional requirements, resistance to drugs, or behavior on reinoculation into animals. That diversity may in part reflect a heterogeneity of cell types in the original culture; however, similar variations have been observed in clones of a single cell. Several workers (347, 348) have described the sudden emergence of new cell types in established cultures, again suggesting somatic mutation as at least one important mechanism leading to cellular variation in culture. The possibility that sudden changes in the properties of a culture may in some instances be referable to its contamination with another cell strain is discussed in a following section.

Within strains of cultured cells, and even within clones, wide differences have been noted in the karyotype of individual cells (349 to 356). Whether intragenic changes occur with equal frequency is not clear. It must, however, be emphasized that such chromosomal changes are not a necessary consequence of even prolonged cultivation (103, 357, 358, 358a), and the role of the serum protein of the medium in causing or preventing chromosomal and nuclear changes (357, 358a; 359 to 361) remains to be clarified.

The development of polyploidy, heteroploidy, and of gross chromosomal aberrations has not been regularly accompanied by demonstrable changes in the nutritional requirements or metabolic activity of the cells. Conversely, however, in two careful studies, Vogt (362, 363) has found that variant clones differing from the parent strain in their serum requirement, morphology, resistance to aminopterin, or resistance to virus, varied so regularly in karyotype from the parent culture as to suggest a positive and perhaps causal relationship between the chromosomal aberrations and the observed phenotypic changes. No such association with a microscopically visible chromosomal abnormality has yet been demonstrated for most of the mutant cell cultures discussed in the following paragraphs, perhaps because it has not regularly been sought; this may be a fruitful area for further investigation. A similar association between karyotypic and phenotypic changes in tumors has been discussed by Hauschka (364) and Klein & Klein (365).

Despite occasional reports that cells deriving from normal tissue mutate in culture to cells that are characterized as malignant (349; 366 to 371), it is debatable whether this is a necessary or regular consequence of their serial propagation *in vitro* [(348, 372, 373); cf. also page 630]. Moreover, no clear relationship has been established between gross chromosomal alterations and the malignant transformation *in vitro*.

The availability of a simple technique for obtaining clones (374, 375) makes it feasible to isolate biochemical mutants. DeMars & Hooper (237) have recently described a method for the selection of mutants which, analogous to the penicillin technique with bacteria, is based on the selective survival of mutant, non-growing cells in a medium containing a cytotoxic agent.

To date, however, there is no counterpart in the case of animal cells to the galaxy of auxotrophic mutants which have been so fruitfully explored in bacteria and molds. The degree to which the polyploidy and heteroploidy characteristic of most mammalian cell cultures contribute to their metabolic stability remains to be determined; it is possible that euploid cultures (103, 358, 376) would be more productive of such mutants. Even in this case, obviously, recessive mutations would not be phenotypically expressed.

A number of cell variants have been described; however, their genetic basis has not been explored in detail, except for the human galactosemic cell cultured by Krooth & Weinberg (159). In this instance, the enzymatic deficiency associated with a specific gene mutation was demonstrable in culture (158). Biochemically uncharacterized variants have been obtained of a rabbit fibroblast culture and were able to grow on a medium inadequate for the bulk of the population (377). Variants have been isolated from a mouse fibroblast (80) and human cells (79, 81) capable of using xylose. Human cell variants have similarly been isolated which, unlike the parent strain, can utilize lactate in lieu of glucose (79) or can grow without exogenous inositol (69). Although the Jensen sarcoma cell has an anomalous requirement for asparagine (51), sublines were isolated which, like most mammalian cells, have no need for exogenous asparagine (52). Clonal differences in the serum requirement of a human cell have also been described (378); a subline of the HeLa S3 clone proved incapable of adaptatively increasing its glutamine synthase activity on exposure to high levels of glutamic acid (6).

Variants have been obtained of a mouse fibroblast (109, 115, 116, 117) and of human cells (105, 112, 118) capable of growing in a protein-free medium which did not support the growth of the parent strain. It is not yet clear whether this reflects a process of adaptation or the selective multiplication of relatively rare mutants. The significance of these observations with respect to protein requirement is for the time being difficult to assess because, under proper experimental conditions, entire cell populations, rather than rare mutants, will similarly grow in a protein-free environment without prior adaptation (119 to 121).

Although a number of biochemical variants, some of which may be mutants, have been described in cell cultures, the phenotypic expression of mutation has usually involved changes in cellular morphology, resistance to drugs, and resistance to viruses. Puck, Cieciura & Fisher (379) have isolated fibroblastic variants from HeLa cell cultures. Dietel & Edlinger (380) have similarly obtained fibroblastic variants from a normally epithelioid culture of human liver cells. A HeLa cell culture which became fibroblastic simultaneously acquired the capacity to hydroxylate phenylalanine to tyrosine (46).

With respect to drug resistance, isolates have been obtained from various cell lines which are resistant to aminopterin (231, 232, 363), amethopterin (381, 382), 2-6-diaminopurine (160), 6-mercaptopurine (160, 231, 232, 383), puromycin (234), 8-azaguanine (233, 234), and 2-deoxyglucose (193). Some of these variants were shown to be mutants, occurring with a frequency of

10^{-4} – 10^{-6} /cell/generation (384, 385). In several instances, a biochemical basis has been suggested for the observed drug resistance. Variant monkey kidney cells resistant to 2,6-diaminopurine and 6-mercaptopurine differed from each other in that the former had no detectable adenosine monophosphate pyrophosphorylase and could utilize hypoxanthine for purine synthesis but not adenine. Conversely, the mutant resistant to 6-mercaptopurine had no detectable inosine monophosphate pyrophosphorylase and could utilize adenine for purine synthesis but not hypoxanthine (160). Davidson (383) found that 6-mercaptopurine was metabolized by strain L-1210 leukemia cells to the corresponding ribotide, which then blocked the conversion of inosine to adenosine monophosphate. In strains resistant to this drug, however, the capacity to form the ribotide was sharply reduced, perhaps explaining the observed resistance. A number of cell lines have been isolated with stable heritable differences in susceptibility to poliovirus (362; 386 to 388). The biochemical basis of these differences is presently unknown.

Fedoroff & Cook (389) have isolated a subline of mouse fibroblasts resistant to normally toxic sera; it further differed in morphology and karyotype from the parent strain. From the same parent culture, a variant was isolated that had a significantly greater resistance to the lethal effects of irradiation, measured in terms of the proportion of cells surviving 1000 r, and that was stable for at least 20 generations (390).

BIOCHEMICAL PROPERTIES OF CANCER CELLS

Although a number of morphologic and biologic criteria have been suggested to differentiate normal and malignant cells (391), no regular and reproducible biochemical difference has yet been found between serially propagated cell cultures deriving from normal and from malignant tissues. Warburg [cf. (392)] noted a high rate of aerobic glycolysis and a relatively low rate of respiration in tumor tissues and suggested that cancer involved an underlying defect in respiratory metabolism. In serial cultures, however, no differences are apparent in these respects between cells deriving from normal and malignant tissues (32, 177, 179, 191). Although Leslie *et al.* (194) have reported that insulin stimulated glycolysis in a malignant cell but not in an embryonic cell, this response has not yet been shown to be a specific property of malignant cell lines.

In possible explanation of the fact that serially propagated cells originating from normal and malignant tissues are biochemically indistinguishable, it has been variously suggested (a) that malignant cells lose the property of malignancy in culture, (b) that normal cells regularly or frequently become malignant in culture [e.g. (370, 371)], or (c) that both normal and malignant cells change on serial culture to a "dedifferentiated" cell which is neither normal nor malignant but is simply a cultured cell.

With respect to the first possibility, Warburg *et al.* (393) did find that, in primary kidney cultures, anaerobic glycolysis increased tenfold within two weeks with the concomitant appearance of aerobic glycolysis. On the basis of his thesis that cancer cells differ from normal in their impaired respiration

and relatively high glycolysis, this could be interpreted to reflect the beginning transformation of normal cells to malignant in the culture. It could, however, mean no more than a changing metabolic pattern as the cells adjust to the markedly different conditions of *in vitro* growth, with a vastly increased growth rate in consequence of the removal of the control mechanisms operative *in vivo*. Although it has been abundantly demonstrated that cells may change in culture, both morphologically and with respect to karyotype [(349 to 356; 362, 363, 394), cf. p. 627], there is no clear evidence relating those chromosomal changes to a "malignant" transformation (cf. 395, 396). Such chromosomal changes also develop in strains originating from malignant tissue (394); conversely, cancer cells in culture can be euploid (397). The fact that serially propagated cells originating from normal tissues may cause the appearance of "tumors" when injected into cortisone-treated or irradiated animals (346, 398) has no necessary implications with respect to a malignant transformation. Such animals, with their normally operative defense mechanisms impaired by that previous treatment, may simply provide a favorable environment for the growth of cells, whether normal or malignant.

Although there is reason to doubt that cells deriving from normal tissue regularly became malignant in culture (cf. below), there have been several clear instances of such transformation. Sublines from the same cloned stem culture may differ markedly in their ability to produce tumor on inoculation in mice (366 to 369), with a suggestive correlation between the tumor-producing activity of the sublines and their glycolytic activity in culture (399). There were also characteristic chromosomal differences between the high and low sarcoma-producing lines, but "there was no present evidence that these differences were directly responsible for their distinctive physiological properties" (396). Conversely, although cells cultured from malignant tissue have occasionally become non-malignant (400), such cells, even after prolonged and rapid serial propagation, usually remain capable of initiating a tumor *in vivo* which is morphologically indistinguishable from the parent strain (1, 102, 372, 373, 401, 402).

The possibility that the biochemical similarity between normal and malignant cells is attributable to their common "dedifferentiation" in culture is at variance with the fact that the nutritional requirements and metabolic activities of such serially propagated cells are, in general, the same as those of cells tested in their very first subculture (37). But by far the most significant evidence yet adduced against the thesis of a regular change in cultured cells with respect to malignancy, and particularly against the thesis that cultured cells regularly become malignant, is provided by the studies of Foley and co-workers (372, 373). On inoculation into the cheek pouch of normal hamsters, cells deriving from normal tissues produced a "tumor" only with inocula of 10^4 - 10^6 cells, and that tumor regressed spontaneously and could not be transplanted; however, cells deriving from malignant tissues produced tumors with inocula of 10 - 10^4 cells, and these tumors were invasive and transplantable.

The failure to demonstrate a biochemical difference between cultured cells

originating from normal and cancer tissue, despite the fact that they are distinguishable in terms of their biological behavior *in vivo*, may mean only that the metabolic differences which almost certainly exist are more subtle than, for instance, a difference in growth requirements. It is also possible that those biochemical differences are simply not expressed under the conditions of *in vitro* cultivation when both types of cells are growing rapidly in a relatively simple environment. Essentially no limitations are then placed on growth other than the elaboration of toxic metabolic products which are removed with each change of medium. Those postulated biochemical differences may, however, find expression in terms of differential growth rates *in vivo*, where different cell types compete for metabolites and where there are cellular interactions that have no counterpart under ordinary culture conditions *in vitro* (332, 333, 403).

MISCELLANEOUS

As pointed out in the introduction, the many studies on the effects of radiation on cultured cells are for the most part beyond the scope of this review. Several reports dealing with the metabolism of cells subsequent to irradiation, however, are pertinent. An x-ray dose of about 500 r leads to a mitotic block, but synthesis of DNA, RNA, and protein continue at a reduced rate for a variable period of time (404 to 408). The continued synthesis of the macromolecules in the absence of cell division presumably leads to the formation of the characteristic multinucleated postradiation giant cells (170, 406, 409). There is a report of increased $P^{32}O_4$ incorporation into the major phosphorus-containing fractions of the cell, particularly into phospholipids, following x-ray irradiation (410).

Two primarily technical matters deserve brief mention. It has become apparent that many, and perhaps most, serially propagated cell lines maintained by the usual techniques in media containing antibiotics are contaminated with both L forms and pleuropneumonia-like organisms. There is ordinarily no evidence of the presence of these organisms, which require special cultural techniques for their demonstration. It is probable that many of the experiments described in this review were inadvertently performed with cultures carrying pleuropneumonia-like organisms or L forms, and it is of course possible that some of the observed metabolic activities were referable to the contaminant rather than to the host cells. To the reviewers, however, it seems unlikely that any substantial revision of the findings here reported will prove necessary on this account. Several procedures for ridding cultures of pleuropneumonia-like organisms have been proposed, but at the present writing their relative effectiveness remains to be assessed (411 to 414).

A second technical problem is the contamination of one cell line by another in the course of their laboratory manipulation. A few cells inadvertently introduced into a culture of a different cell line may have a selective advantage and outgrow the original cells, but the effective substitution of one line by another may be masked by their morphologic and metabolic similarity. A number of such mishaps (352, 353, 415, 416) have already been reported.

Since many, if not all, cell lines can be preserved indefinitely at -70° after being frozen in a medium containing glycerol (417, 418), it is possible, and obviously desirable, to maintain standard reference cultures in this manner.

The presence in serum of growth-promoting as well as growth-inhibitory factors has been discussed in a preceding section. Antisera can be produced to cultured cells which are highly cytotoxic, particularly with added complement (416; 419 to 426), and which will react with the corresponding cells in complement fixation (416, 421) and hemagglutination (426, 427) procedures. A beginning has been made in unravelling the antigenic relationships between different cell lines and in the understanding of the relative importance of species, organ, and specific cellular antigens as determinants of specificity (416; 419 to 427).

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BIOCHEMISTRY OF GENETIC FACTORS^{1,2}

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INTRODUCTION

Since the classical work of Beadle & Tatum (1), it has been generally accepted that genetic information is expressed through control over the structure of proteins. It is known that when several mutations affect the same enzymatic activity they are closely linked on the genetic map and that these mutations can alter the structure of the enzyme molecule. The term "gene" is now most commonly used to describe that localized region of the genetic structure (now thought to be DNA or RNA) in which mutations affect a particular protein or a particular function of the organism.

With the increased genetic resolution possible in crosses of microorganisms, it has been shown that mutations which affect the same or different genes, can be arranged in a linear order on a genetic map (2 to 5). Most of these studies have utilized two- or three-factor recombinational analysis and have yielded results quite similar to those obtained for intergenic recombination in higher organisms, i.e., an unambiguous one-dimensional ordering of mutations and at least approximate additivity along the genetic map. Benzer (6) reported the results of crosses, between 145 deletions in the r_{II} region of the phage T4, which fell into about 45 different classes. The data were shown to be consistent with a one-dimensional ordering along the genetic map.

The hypothesis that the linear genetic order is directly related to the linear structure of the nucleic acids seems plausible and has been widely assumed for years, but there is at present no experimental evidence to support it. On the other hand, a more general correspondence between closeness on the genetic map and closeness on the DNA molecule is experimentally justified by work with transformation and with the timing of the transfer of genetic markers from the male to the female in bacterial crosses [see Hayes (7)]. Studies by Hotchkiss & Lacks (8) on the enzyme amyloamylase in pneumococcus have shown that mutations in the same gene are carried on a relatively small piece of DNA. Some of the mutations behaved genetically as points whereas others behaved as though they occupied a region of the genetic map. The latter were more easily inactivated at high temperatures than were the former. Thus, whether or not the relationship is strictly linear, there does seem to be strong experimental support for the hypothesis that the gene consists of a contiguous stretch of DNA.

CHEMICAL AND PHYSICAL PROPERTIES OF NUCLEIC ACIDS

Nucleotide sequences.—Although the determination of the base sequences of the nucleic acids is of the utmost interest and Khorana (9) and Dulbecco

¹ The survey of the literature pertaining to this review was concluded on October, 1, 1960.

² The following abbreviations are used: DNase for deoxyribonuclease; RNase for ribonuclease; TMV for tobacco mosaic virus.

& Smith (10) have described methods of identifying the terminal group of polynucleotide chains, the stepwise analysis of the sequences appears presently to be impracticable. Instead, growing attention has been directed to release of oligonucleotides by selective enzyme or chemical attack and identification and estimation of the products. It is hoped that such studies may indicate broad patterns of nucleotide sequences. Rushinzy & Knight separated and estimated the oligonucleotides released from nucleic acids by enzyme digestion (11, 12, 13). These investigators demonstrated differences between the RNA of two strains of TMV (14). Reddi (15) elaborated upon this technique by treating the RNase digest with a second enzyme which released the adenylic acid sequences.

Burton & Petersen (16, 17) degraded DNA prepared from eight different organisms by digestion with diphenylamine in formic acid solution. The purine bases were removed by this treatment, and the pyrimidine sequences present in the original DNA were released; each end of the sequence terminated in a phosphate group. When these terminal groups had been removed by phosphomonoesterase, many of the remaining oligonucleotides were separated by two-dimensional chromatography and quantitatively determined. The sequences of bases were shown to be different for each organism and statistically non-random. These authors found a distinctly larger amount of monopyrimidine fragments in calf thymus DNA than had been found by Shapiro & Chargaff (18). A resolution of these differences must await further work. Lichtenstein & Cohen (19) studied the mixture of nucleotides released by DNase and snake venom diesterase digestion of DNA from various bacteriophage mutants. They failed to detect any significant differences between the DNA from the original and mutant viruses. Shapiro & Chargaff (20) released pyrimidine nucleotide sequences from rye germ DNA by controlled acid hydrolysis and showed that the bases were arranged in a non-random way. They also found that the amount of guanine in moles per cent was equal to the sum of 5-methyl-cytosine and cytosine, also in moles per cent, as would be expected if either of the two latter bases could form equivalent hydrogen bonds with guanine in the way pictured by Watson & Crick (21). 5-Methyl-cytosine did not randomly replace cytosine but was found more frequently in "solitary" positions between purine bases. The DNA was separated into fractions with different ratios of adenine plus thymine to guanine plus cytosine plus 5-methyl-cytosine; the ratio of 5-methyl-cytosine to cytosine was not constant among these fractions. Shapiro & Chargaff (20) point out that if, in the course of synthesis, differentiation is made between cytosine and 5-methyl-cytosine groups, a selection process must be involved beyond that of the hydrogen-bonding requirements. However, we are aware of no evidence that 5-methyl-cytidylic acid is incorporated as such into DNA. It is therefore possible that the cytosine groups are methylated after incorporation into the DNA. If the neighboring bases influence the activity of the methylating enzyme, a selective methylation could be observed. A precedent for such a scheme exists; Kornberg *et al.* (22) have shown that glycosylation

of hydroxymethyl-cytosine is accomplished after the base has been incorporated into the DNA.

Studies on nucleic acid hydrolyzates are presently limited by the resolving power of chromatographic and electrophoretic methods for separating oligonucleotides. Further progress in sequence analysis will also depend upon the isolation and characterization of more degradative enzymes (23 to 27).

Josse & Kornberg (28) have developed methods for determining the frequency of neighboring pairs of bases along a polynucleotide chain synthesized by the polymerase system. They have shown that the newly synthesized DNA has the same non-random distribution of neighboring bases and the same composition as the primer DNA. This is the clearest demonstration to date that in this *in vitro* system the new DNA chains are synthesized by use of the primer as a template. By an elegant analysis of the frequency of neighboring bases, these authors provided the first chemical evidence that the polynucleotide chains in DNA are antiparallel, as suggested by Watson & Crick (21).

Size of the DNA molecule.—The recent demonstration that all the observed genes of the bacterium *Escherichia coli* [Wollman & Jacob (29)] and of the phage T2 [Sekely (30); Streisinger & Bruce (31)] lie on a single linkage group indicates that these organisms have a physically continuous entity corresponding functionally to a chromosome. Because physical-chemical evidence shows that DNA molecules have a molecular weight of the order of six to ten million, it has been suggested [Freese (32); Kellenberger (33)] that chromosomes consist of pieces of DNA hooked together by some type of non-DNA linkers. Since very large pieces of DNA tend to remain together during multiplication in phage, bacteria, and the chromosomes of higher organisms [(Levinthal (34); Taylor (35); Forro & Wertheimer (36))], the linkers would have to have properties that would allow them to hold at least single chains of DNA together during cell division.

However, recent demonstrations of the great fragility of DNA of very high molecular weight have cast doubts on the significance of the molecular weights of DNA as isolated by most of the usual procedures. Anderson and co-workers found that a solution of isolated cell nuclei in strong saline exhibits a very high viscosity which is diminished by shear forces, DNase (37), and radiation (38). They suggested that this high viscosity is attributable to DNA structures much longer than those present in solutions of purified DNA. Davison (39), taking precautions to minimize shearing forces during the isolation procedure, obtained DNA with an exceptionally high sedimentation coefficient. Shearing of these solutions through a hypodermic needle of the type commonly used for loading ultracentrifuge cells gave a DNA with a distribution of sedimentation coefficients closer to that seen in conventional preparations. Cavalieri & Rosenberg (40) also reported that high shearing forces can lower substantially the molecular weight of DNA as isolated by some of the usual methods. Rosenberg & Bendich (41) and Kit (42) showed

that degradation of DNA occurred under the shear forces imposed by the tissue homogenizers commonly used during DNA purification.

As the shear forces are increased, the molecular weight of the resulting DNA drops with no evidence of any natural breaking points. It has been demonstrated theoretically [Levinthal (44)] that the magnitude of the force developed along a DNA molecule by velocity gradients common in laboratory manipulations would be sufficient to break the phosphate ester links at the DNA backbone. Since the maximum force increases as the square of the molecular weight, the isolation of extremely long pieces which are still intact rapidly becomes prohibitively difficult. The theory also shows that non-random breaks produced by shear degradation would lead to a narrow molecular weight distribution. In contrast, sonication of DNA yields material with a wide molecular weight distribution (45).

Studies on DNA are therefore compatible with the possibility that DNA occurs *in vivo* in the form of very long molecules which are subject to random scission in the procedures used for purification. It is possible that even the DNA in a mammalian chromosome may consist of a single molecule with a contour length of several cm. It has been pointed out that, despite the enormous length of this molecule, the problem of unwinding of the two chains of the DNA molecule would not be prohibited, at least by energetic considerations, if the synthesis of the new material went on simultaneously with the separation of the two chains of the old (44). It may thus be unnecessary to postulate the existence of any linkers, and certainly there is no concrete evidence for them. If, on the other hand, the two chains of such giant molecules were separated by a change in the intracellular environment which reduced the hydrogen-bond energy to zero, linkers with very special properties would be required, since the unwinding rate would otherwise be too low [Longuet-Higgins & Zimm (46)].

The occurrence of random breaks in DNA during isolation would explain the lack of reproducibility between different DNA preparations and the chromatographic heterogeneity of specific activities in transforming principle [Beiser *et al.* (47)]. The observed dependence of the molecular weight of the DNA on the method of preparation makes the reported correlation between the sizes of RNA and DNA (48) of dubious significance.

Experiments designed to correlate intrinsic viscosity $[\eta]$ and molecular weight M_L (measured by light scattering) of a number of DNA preparations were reported by Lett & Stacey (49). They found that

$$[\eta] = 1.1 \times 10^{-5} M_L,$$

and they noted that any deviation from this behavior could be indicative of damage to the DNA structure, for example, by irradiation. The DNA samples studied ranged up to $M_L = 10^7$.

Eigner (50) reviewed a large number of light-scattering measurements on DNA, and from parallel sedimentation and viscosity studies he deduced the relationships between S_{20} , $[\eta]$, and M_L . From these studies he found a definite

curvature in both the $\log S_{20}$ vs. $\log M_L$ and $\log [\eta]$ vs. $\log M_L$ plots. He calculated from these data the size of the parameter β in the Flory-Mandelkern equation (51) and found a molecular weight dependence. He concluded that molecular weights for DNA can be computed most simply in the range of high M_L from $[\eta]$, S_{20} measurements, and the empirical value of β .

In the range of M_L smaller than 10^7 , the equations of Eigner are very similar to those of Doty *et al.* (45). If these equations are extrapolated, however, they diverge progressively; for example, a material with $S_{20} = 50$ would have $M_L = 43 \times 10^6$ from Eigner's relationships but 68×10^6 from Doty's.

Lett & Stacey's results agree broadly with Doty's equation:

$$[\eta] = 1.45 \times 10^{-6} M^{1.12}.$$

The numerical differences may be related to the fact that Doty measured $[\eta]$ by extrapolating to zero shear from η_{sp} measured at shears of 20 to 30 sec^{-1} , whereas the measurements reported by Stacey were extrapolated from 2 sec^{-1} .

A broad agreement exists among the equations derived by Lett & Stacey, Doty *et al.*, Eigner, and Cavalieri & Rosenberg (40) for the relationships between $[\eta]$, S_{20} , and M_L for native DNA. Butler and co-workers (52) found a similar correlation between S_{20} and $[\eta]$ for a number of samples, but the values they determined for M_L were not simply related to either of these properties. These authors questioned the applicability of the light-scattering method to molecules with such large linear dimensions as DNA of molecular weight 8×10^6 would appear to have. Hermans (53) has reported a poor correlation between $[\eta]$, S_{20} , and M_L for a large number of DNA preparations, and he demonstrated that the scatter of the results could be improved by treating the DNA with chymotrypsin to remove contaminating protein. DNA must therefore be carefully purified before a reasonable correlation between the molecular weight and the physical properties of the solution can be observed.

The reliability of the determinations of M_L between $M_L = 5 \times 10^6$ and 10×10^6 remains in dispute. Rice & Geiduschek (54) discussed the difficulties of the method but concluded that the measurements were valid despite a concealed curvature in the Zimm plot, which made uncertain the extrapolation which is necessary to obtain the value of $1/M_L$, and despite also the complication of an unknown degree of polydispersity. Cavalieri & Rosenberg (40) have also concluded that the uncertainties introduce no greater error than is inherent in the light-scattering method. The agreement between the independent studies mentioned above appears to show that the method is applicable up to $M_L = 8 \times 10^6$. On the other hand, Doty *et al.* (55), like Butler *et al.*, have found inconsistencies above $M_L = 5 \times 10^6$, so there must remain some doubt about the upper range of the equations which relate S_{20} and $[\eta]$ with M_L , and any extrapolation of the equations beyond $M_L = 10^7$ is clearly unjustified. This situation is unfortunate, since at this time DNA that is apparently much larger is being studied, and there is available no absolute method of molecular weight determination. The method of equilibrium

centrifugation in a cesium chloride density gradient (56) cannot be confidently applied, since the experiments of Sueoka, Marmur & Doty (57, 58) and Meselson & Rolfe (59) have demonstrated a density heterogeneity in different DNA samples, and Sueoka (60) has shown that for this reason the molecular weight determined by banding can only be minimal. Furthermore, an unknown error may arise from solvation of the DNA in the concentrated cesium chloride solution (61).

In the study of DNA isolated from bacteriophage, problems have arisen in the assignment of very high molecular weights. Levinthal (34) and Levinthal & Thomas (62) found by autoradiographic experiments that the DNA extracted from the phage T2 by osmotic shock consisted of one fraction of molecular weight about 50×10^6 , representing some 40 per cent of the DNA of the intact virus, the remainder of the material being smaller. Thomas (63) found that the smaller material had a molecular weight of approximately 12×10^6 . Doubts about these results arose when studies on similar material by sedimentation equilibrium and sedimentation velocity showed a homogeneous preparation of molecular weight 12 to 14×10^6 [Meselson *et al.* (56); Fleischman (64)]. However, Brown & Brown reported the isolation of two fractions from T2 DNA by chromatography (65). One of these fractions they identified with the large subunit, and they noted that this appeared to be unstable. Thomas (63) also reported that the large subunit could be readily broken down into smaller pieces by treatments commonly used to remove protein from DNA preparations. Davison (43) then showed that the sedimentation coefficient of the DNA in a "shocked" solution of T2 phage was drastically lowered if the solution was subjected to shearing forces, which, it is believed, degrade the molecule by double-chain scission. The undegraded DNA described by Davison had a sedimentation coefficient far higher than those measured previously on DNA, and no reliable molecular weight can be ascribed to these molecules. However, it was shown that, if the smaller molecules detected by Thomas (63) had sedimentation characteristics similar to those of normal DNA, they probably would have been resolved in the sedimentation diagrams. It seems possible that the material examined by the autoradiographic method was partially degraded by shear, whereas the older hydrodynamic studies were done on more uniformly degraded material. It is also possible that the DNA molecules could be folded in some way which would make their hydrodynamic behavior anomalous. Thomas & Matheson (66) reported that the viscosity of T2 phage DNA is lower than would be predicted from a mixture of large subunits and smaller particles. These results could also be explained if the large subunits or other pieces were folded or if these workers had unwittingly degraded the DNA by shear forces.

Hershey and co-workers (67, 68) described a chromatographic method for differentiating the DNA released from shocked T2 phage from that degraded by stirring. They showed that progressively faster stirring produced discrete chromatographic peaks which were thought to be fragments of the original molecules, successively halved in accord with the assumed mechan-

ism of shear degradation (44). Hershey & Burgi (68) found that the DNA released by phenol became more sensitive to shear degradation when the concentration was lowered. The mutual protection was lost at concentrations between 4 μ g. and 0.4 μ g. per ml. Since sedimentation studies using ultraviolet optics in the ultracentrifuge have not been conducted below concentrations of 6 μ g. per ml., it must be concluded that intermolecular interactions may occur in these solutions, and it cannot be assumed that a valid extrapolation of S_{20} to zero concentration can be made from the sedimentation results thus far obtained. Even if it were reasonable to extrapolate the equation of Doty *et al.* (45) or Eigner (50) to obtain the value of M from S_{20} , the latter value is not available. Hence, the number of DNA molecules in T2 phage is not known, although Hershey & Burgi (68), from the homogeneity of the chromatographic peak of the DNA first isolated, believe that all the molecules released from the phage are of identical length.

The sedimentation behavior of DNA and the mechanism of the self-sharpening of the boundaries have been investigated by Coates & Jordan (69). Schumaker & Marano (70) have reported the appearance of a variable number of discrete peaks in the sedimentation of DNA solutions at high pH, but it is possible that this behavior reflects some unrecognized boundary anomalies rather than the differentiation of some families of DNA molecules. Further reports of x-ray diffraction studies on DNA (71, 72) have confirmed the general features of the structure proposed by Watson & Crick (21).

A number of spectroscopic studies on DNA have been reported. Inman & Jordan (73), having carefully measured the extinction coefficient of native DNA, conclude that $E_p = 6630$. The protonation of the bases during acid denaturation has been studied by Dove *et al.* (74). Bunville & Geiduschek (75) have correlated the critical pH for acid denaturation with the base composition of the DNA. A distinction between the action spectra of single and double-stranded DNA has been shown by Setlow (76). Emanuel (77) has reported a reversible hyperchromicity, not associated with denaturation, in DNA dissolved in certain concentrated salt solutions. Other factors that influence the hypochromicity of the bases have been investigated by Spirin *et al.* (78).

Denaturation of DNA.—Marmur (79) has studied the "melting-out" temperature of DNA from thermophilic organisms and has shown that the DNA is not exceptional but melts out at a temperature corresponding to its base analysis (81). Pullman & Pullman (82) have given theoretical grounds for the experimental observation that the heat stability of the guanine-cytosine hydrogen bonding is greater than that of the adenine-thymine pair.

The denaturation of DNA by acid, base, heat, or organic solutions causes a remarkable change in the physical properties of the polymer. Oth (83) and Inman & Jordan (84, 85, 86) have studied the sedimentation behavior and other properties of DNA denatured at low ionic strength. Bradley & Felsenfeld (87) demonstrated a difference in the binding of acridine orange to native and denatured DNA.

Doty, Marmur, and co-workers have studied the heat denaturation of DNA. The hydrogen bonds are disrupted when DNA is heated and they reform on cooling. In animal DNA the reformed bonds do not have the original specificity (88). On the other hand, bacterial DNA displays differences in properties depending upon whether the solutions are slowly or quickly cooled (55). Physical experiments suggested that in the heated solutions the strands of the double helix separate completely, and, if the solutions are slowly cooled, a considerable number of the strands of bacterial DNA reaggregate with their complementary strands to give "renatured" DNA with properties similar to the native material, whereas rapid cooling causes a random reformation of hydrogen bonds. A striking difference between slow and fast cooling was demonstrated by comparing the fraction of transforming ability recovered from a heated solution of pneumococcal DNA (89). Slow cooling gave a 35-fold greater activity, and, if a rapidly-cooled solution of DNA was "annealed" at a higher temperature again, the recovered activity was markedly increased. Above a certain concentration of DNA, aggregation occurs on cooling the solution of denatured molecules coincident with the formation of intermolecular hydrogen bonds. If, however, the ionic strength of the solution is low, the mutual repulsion of the phosphate groups prevents the formation of these complexes. The addition of salt to the solutions results in aggregation (50).

The separation of the DNA strands during denaturation was reported some years ago by Alexander & Stacey (90). Their conclusions were questioned by Rice & Doty (91) and Geiduschek & Holtzer (92), but it now appears that those conclusions may have been correct, although the evidence was incomplete.

These experiments offer a solution for an anomaly in the physical properties of denatured DNA. When DNA solutions are heated, the viscosity falls sharply, whereas the sedimentation coefficient remains largely unchanged (93). This experiment implies a lowering of the molecular weight. Light-scattering measurements, however, consistently indicate that the weight is unchanged. This result may now be explained if it is assumed that, at the DNA concentration used for light-scattering, aggregation after denaturation fortuitously leaves the molecular weight apparently unchanged.

Cavalieri and co-workers (94, 95) have studied the effect of heating DNA in cesium chloride solutions. Since they claim a specific action for this salt, the work cannot be directly compared with that of Doty *et al.* (55), but the results appear conflicting. Cavalieri *et al.* (94) showed by light scattering that the molecular weight of DNA preparations could be drastically reduced by chymotrypsin treatment or interfacial denaturation. Subsequent heating in CsCl halved the molecular weight. When the product of the reaction was degraded by DNase, the kinetics of the lowering of the molecular weight indicated that the molecule was double-stranded. Cavalieri concluded that DNA consists of double helices paired by heat-labile bridges.

In other studies a number of workers (39, 53, 96) have used chymo-

trypsin on their DNA preparations without observing the large drop in molecular weight reported by Cavalieri unless the preparations were grossly contaminated by protein. Butler *et al.* (96) showed that the enzyme primarily affected the rapidly sedimenting material. If this material were laterally cross-linked with protein, its removal would be expected to lower the Z-average molecular weight (which is heavily weighted by the larger molecules) with little change in the radius of gyration. In addition, the DNase digestion kinetics do not preclude the separation of the strands on heating: the decrease of ultraviolet absorption on cooling shows that a large fraction of the transverse hydrogen bonds reform (88), and thus, the product of heating would be expected to be double-stranded.

Levine *et al.* (97) demonstrated the antigenicity of heat-denatured phage DNA, and more recent work has suggested that the antigenicity is related to the glucosylation of the hydroxymethyl-cytosine. Grossman *et al.* (98) have studied the reaction of formaldehyde with native and denatured DNA.

Preparation and fractionation.—Sadron *et al.* (99) have reported the preparation of quaternary ammonium salts of RNA and DNA which are soluble in organic liquids.

Details of the phenol method of preparing DNA (100, 101) and RNA (101, 102) have been published and the separation and fractionation of the two acids by electrophoresis (103), selective precipitation (104), chromatography (105, 106, 107), and countercurrent fractionation (108, 109) have been described.

Analysis of the bases in RNA and DNA from a large number of plants was reported by Belozerskii *et al.* (110, 111). They detected 5-methyl-cytosine in each DNA sample. Watson & Littlefield reported the isolation and properties of DNA from the Shope papilloma virus (112).

Physical properties of RNA.—Since this review is concerned with the genetic materials and evidence is growing that the RNA of the cell comprises many fractions, only some of which may be related to the transfer of genetic information, the following discussion will be limited to viral RNA.

The relative instability of RNA from TMV has prompted several investigations. Friesen & Sinsheimer (113) related the loss of infectivity of the RNA to degradation to smaller particles. On the other hand, Spirin *et al.* (114, 115, 116) found little evidence for chain scission when the RNA was at room temperature, but a drop in the viscosity of the RNA at elevated temperatures could be correlated with the loss of infectivity. It remains to be seen if this lability indicates discontinuities in the RNA strand or if degradation results from contamination by RNase (117).

The physical properties of RNA (118) are very similar to those of the single-stranded DNA from phage ϕ X 174 (119) and denatured DNA. The hypochromicity of RNA has been measured, and it has been concluded that many intramolecular hydrogen bonds are present at normal temperatures (116). Fresco & Alberts have suggested that the hydrogen bonds could give rise to random stretches of double helix in which complementary bases asso-

ciate while bases that cannot bond with the opposing strand form loops directed out from the helix (120).

Synthetic polynucleotides.—A number of investigations on synthetic polynucleotides have been reported. Rich (121), who has reviewed the earlier work in this field, pointed out that these studies are of importance in defining what interaction may occur between the corresponding biological polymers rather than what necessarily does take place. The demonstration of the formation of a triple polynucleotide helix prompted the postulate that cytoplasmic RNA might be synthesized in this fashion on double-stranded DNA. Rich's later report (122) of the formation of a double helix between a polyribo- and a polydeoxyribonucleotide strand has naturally introduced the idea that the information may be imparted by a single strand of DNA. Lipsett (123) has reported helix formation in a polyuridylic acid solution at low temperatures. It has also been shown that the complexes between high molecular weight polyuridylic acid and short polyadenylic acid sequences melt out at temperatures which increase with the length of the latter polymer (124). Davies isolated a crystalline polymer complex between antiparallel chains of polyinosinic and polycytidylic acids (125).

Replication of DNA.—The specificity of the polymerases described by Kornberg (126) and others [see discussion by Davidson (127)] makes it likely that these enzymes are involved in DNA synthesis *in vivo*. Since the enzymes can utilize single-strand DNA to prime the synthetic reaction—the calf thymus enzyme exclusively so (128)—it is probable that DNA synthesis proceeds along each single strand of the double helix, perhaps in the manner postulated by Levinthal & Crane (129) in which the daughter strands grow as rapidly as the parent helix unwinds.

Atwood (130) points out that at least some of these polymerases can utilize only the 3'-nucleotide triphosphates for substrates. Hence, he deduces, the enzyme can couple the nucleotides along only one direction and therefore only along one of the antiparallel DNA strands as the helix unwinds. We feel that the logic of this argument is faulty. Since the specificity of the pairing bases is dictated by the priming strand, it seems likely that the first stage of the laying down of a new DNA strand is the pairing of the nucleotide triphosphates with their future complementary bases on the primer strand. The polymerase probably then links the adjacent bases with the elimination of pyrophosphate. If this is the synthetic mechanism, there is no *a priori* reason why the enzyme should differentiate the directions in space of the respective base neighbors on either strand.

EXPRESSION OF GENETIC INFORMATION

The gene is a linear element, and the only portion of a globular protein which could have a corresponding linearity seems to be the amino acid sequences in the polypeptide chains. It has therefore been postulated that nucleotide sequences on DNA or RNA dictate the identity and sequences of amino acids in proteins. The way in which this information is encoded in the

base sequence has become known as the "coding problem." Some recent papers have discussed the requirements of the genetic code, the experimental facts to be explained, and the deficiencies of present theories (131, 132, 133). Since fewer bases than amino acids have been detected, a one-to-one code is not possible, and each amino acid must be defined by several bases. To this point, agreement is general, but real progress must await further experimental data.

The nature of observable mutations.—A corollary to the hypothesis that DNA dictates the formation of a linear sequence of amino acids is that the folding of the polypeptide chain to form the secondary and tertiary structure of the protein must follow uniquely from the sequence of the amino acids and, furthermore, that there should be no special folding genes required in the making of a protein. Experimentally, however, the only data available at present indicate simply that the mutations which affect a particular protein are localized and that many of these mutations can cause the production of an altered protein (134). (Such alterations may be recognized by changes in the turnover number, the Michaelis constant, and inhibitor constants of an enzyme; and in the heat stability, activation energy, and electrophoretic mobility of a protein.) Although, in several cases, mutations have been shown to result in the change of a single amino acid in a protein, the corresponding analyses for mutations in which the fine-structure genetics can also be carried out is just beginning.

It is difficult to evaluate the significance of the various classes of mutants detected after mutagens have modified the DNA of different organisms, since the type of mutations observed depends on the methods of selection and the complexity and function of the protein, as well as the properties of the genetic code itself. Because a number of nucleotides are needed to specify each amino acid, an alteration in any base pair by a mutagen raises the possibility of a class of so-called nonsense mutations, as well as a class of mis-sense mutations (135). A mis sense mutation in this context is one which causes a substitution of one amino acid for another at a particular position in the protein. However, if not all sequences of bases correspond to amino acids, then a mutation could result in a sequence which did not correspond to an amino acid and which, in turn, could lead to the production of either smaller peptide chains or something in no way recognizable as related to the original protein. In many of the genes which have been studied, mutations leading to a total loss of enzymatic activity still leave the organism viable if it is grown in a properly supplemented medium. In these cases, it has been found that a significant fraction of the mutants observed make little or no immunologically cross-reacting material. On the other hand, not all these mutants that are not immunologically cross-reacting could correspond to nonsense mutations, since the effect of some of them can be reversed by a suppressor mutation which occurs outside the primary structural gene. In the case of tryptophan synthetase in *E. coli*, Yanofsky (3) reported suppressor mutations located outside the gene which restored the activity of cross-reacting

negative cells. He also found a case in which a mutation produced an altered protein, which was recognized as different by its electrophoretic behavior and its reduced turnover number, and a suppressor mutation, which restored enzymatic activity to the cell, caused the production of electrophoretically normal, as well as electrophoretically altered, tryptophan synthetase protein.

No suppressor mutations outside the structural gene have yet been observed for the alkaline phosphatase of *E. coli*. However, it has been found that the same gene is expressed differently whether it is in one strain of *E. coli* or another (2, 136). Several mutants that were isolated in a male *Hfr* strain and had no detectable enzymatic activity or ability to produce cross-reacting material in this strain, had both when the same gene was transferred by mating to a female recipient strain.

All these anomalies may reflect the fact that more than one genetic region specifically controls the formation of a protein, either by affecting its folding or even by affecting the primary amino acid sequence. However, until such time as a complete analysis of the amino acid composition of these altered proteins is made, it is possible that all the observed anomalies have to do with changes in the ionic environment (including metal ions) inside the cell, and these, in turn, affect the probability that a particular favorable folding of the protein takes place.

Correlation with protein structure.—In many different organisms a large number of mutations have been observed which affect the enzymatic activity of what seems to be a single enzymatic step. Only those studies of the differences in the enzyme protein molecule itself, will be reviewed here. A number of systems are being developed in which it is possible to carry out fine-structure genetics and detailed chemistry on the altered proteins. In bacteria, studies have been reported on the tryptophan synthetase (3) and the alkaline phosphatase in *E. coli* (2, 136). In both of these instances, fine-structure genetic mapping has been carried out either by transduction or by recombination after sexual mating. Mutants have been found in both systems which make an altered protein, as judged by the existence of immunologically cross-reacting material which can be purified with procedures effective for the wild-type enzyme. Tryptophan synthetase of *E. coli* (3) has been shown to be an enzyme consisting of two parts, each of which catalyzes a separate step in the chemical reaction. The two parts seem to be made separately by the cell and can be joined together *in vitro* to form active enzyme. The genes for each of these two protein parts are adjacent to each other on the bacterial chromosome, and one of them, the A-protein, has a molecular weight of 29,500. Several mutants have been found which produce enzymatically inactive A-protein but a full complement of immunologically cross-reacting A material. These mutations are closely linked on the genetic map, and their effect on the amino acid sequence in the protein is being investigated. The alkaline phosphatase of *E. coli* has a molecular weight of 80,000 but is composed of two identical subunits of 40,000 each. In this case also, there are mutants that make a related protein; also many reverse mutations occurring at or

near the site of the original native mutation make a protein that is enzymatically very similar to, but electrophoretically different from, the wild-type enzyme.

Two bacteriophage systems are being investigated, the head-protein of the phage T4 [Brenner & Barnett (137)] and the lysozyme of the phage T4 [Dreyer (138)]. The latter system seems particularly promising since the molecular weight of the protein is only 15,000 and the genetic fine-structure can be determined with high resolution. In neither of these systems have amino acid differences been reported.

Woodward, Partridge & Giles (139) examined the adenylosuccinase formed in mutants and revertants of *Neurospora crassa*. They showed that reversions which appeared to occur at the same genetic locus as the original mutation can produce protein that is different from the wild-type enzyme. Kirkman *et al.* (140) studied the enzyme glucose-6-phosphate dehydrogenase in human mutants. They showed that this enzyme has a different Michaelis constant and a different pH optimum from the wild-type enzyme. This is the first case, other than sickle-cell anemia, in which a genetically controlled human disease has been demonstrated to be associated with an alteration in a specifically involved protein molecule.

A rough estimate of the amount of DNA necessary to code for a single amino acid has been reported (2, 136); this estimate [based on the conversion of recombination length to amount of DNA estimated by Fuerst, Wollman & Jacob (quoted in 29)] was obtained by comparing the recombination length in the *coli* chromosome which corresponds to alkaline phosphatase with the size of the protein itself. The experimental data are consistent with a coding ratio, i.e., nucleotide pairs in DNA to amino acid in the protein, of between 3 and 10.

Amino acid alterations.—The two proteins in which single mutations have been shown to result in the change of one amino acid in the molecule are human hemoglobin and TMV. The former is the protein in which such single amino acid differences were first observed by Ingram (141) for the normal and sickle-cell hemoglobin. Although neither fine-structure genetic analysis nor deliberate mutagenic experiments can be done with human beings, the blood of a large number of individuals is examined for clinical purposes, and several chemically different hemoglobins have been found.

Mutations producing amino acid alterations in either the α - or the β -chain of adult hemoglobin have been observed. Although they do not appear to be spread randomly over the molecules, they do occur in several different peptides from trypsin hydrolyzates (142). Hunt (143) has shown that foetal hemoglobin contains the same α -chain as does adult hemoglobin, whereas the β -chains are similar but have alterations in about 20 per cent of their amino acids. From studies on a person with an alteration in the α -chain, it seems clear that the same gene controls this chain in both adult and foetal hemoglobin (144). An individual with four types of hemoglobin was shown by Baglioni, Ingram & Sullivan to produce both a normal and an altered α -

chain, as well as a normal and an altered β -chain. Each molecule of hemoglobin contained a pair of one or other of the two different α -chains combined with a pair of one of the two β forms. Individuals in the same family have been shown to contain the four hemoglobin types, but the children of these individuals, when married to normal hemoglobin producers, frequently contain only one of the mutated genes and produce only two types of hemoglobin (145). Thus, the gene for the α -chain and the gene for the β -chain are not closely linked. This situation is very different from that found for the A and B protein of tryptophan synthetase, where the two genes are adjacent in the *E. coli* linkage system. Ingram & Stretton (146) suggested that various thalassemias in men may be caused by the reduced amount of protein resulting from mutations in either the α - or the β -chain.

Wittmann (147) carried out a complete amino acid analysis on each of the 12 peptides from trypsin hydrolyzates of the wild type, 14 nitrous acid induced, and 12 spontaneous mutants of TMV. Eight of the spontaneous mutants were selected in such a way as to minimize the probability of obtaining double mutants. Two of them showed single amino acid changes, whereas the others were identical to the wild type. Of the 14 induced mutants, six showed no change, one showed changes in two amino acids, and seven showed single amino acid changes. The fact that mutants exist with an unchanged amino acid composition for each of the peptides is not surprising since the RNA in TMV probably codes for other proteins necessary for viral production in addition to the protein coating the virus itself. The mutant with two altered amino acids could be explained by the fact that prolonged nitrous acid treatment was used and might have induced two mutations in the RNA. Tsugita & Fraenkel-Conrat (148) have induced mutants in TMV by the action of nitrous acid, and in one of them they were able to detect three amino acid changes by careful analysis of the complete virus protein.

An ingenious new method of determining the sequential order of the trypsin-produced peptides of a protein was used in studies with TMV [Wittmann (149)]. Four different strains of the virus have different amounts of arginine or lysine, and the trypsin-produced peptides obtained with different strains, could be used to identify neighboring peptides. In this way, the order of nine of the 12 tryptic peptides was established.

Regulatory mutations.—In addition to the mutations which are known to affect the structure of the proteins that a cell can produce, there are also mutations which affect the amounts of the various proteins that the cell actually produces under given environmental conditions. Many bacterial enzymes have been shown to be produced in large or small amounts, depending on the medium in which the cells are grown (150). In several instances, mutations have been observed which lead to the breakdown of such control mechanisms. In recent years there has been considerable work on the nature of these mutations and the genetic interactions between the control genes and the structural genes.

β -Galactosidase of *E. coli* is made at a high level if the cells are grown in a

medium that contains an inducer (a galactoside but not necessarily a substrate for the enzyme) but does not contain glucose. A constitutive mutation has been studied which is closely linked to the structural gene for this enzyme and which leads to the production of high levels of enzyme without inducer. The structural gene has been designated *z* and the inducible mutation *i*. Pardee, Jacob & Monod (151) studied the kinetics of enzyme formation in zygotes in which the *z*+*i*+genes (i.e., normal for each) are transferred by mating into a cell which cannot make active enzyme but has the inducible mutation. As the *z*+gene entered the recipient cell, enzyme synthesis was observed to commence rapidly without the need for inducer. After about an hour, constitutive production stopped, but the zygote could still form β -galactosidase when inducer was added to the medium. It was therefore suggested that the *i* gene in the normal state was responsible for the formation of a repressor, the action of which is opposed in the normal cell by the addition of inducer. When the genes are transferred into the recipient cell, which does not contain repressor, constitutive synthesis ensues until the *i*+gene has time to make sufficient repressor to shut off this production of further enzyme. For the β -galactosidase control system, only one such repressor gene has been observed; however, the control of tyrosinase in *Neurospora* (152) and of alkaline phosphatase (153) in *E. coli* involves at least two genes. For alkaline phosphatase, one of them is closely linked to the structural gene, and, as with β -galactosidase, it has been shown to be dominant in the normal state. The other gene has been located on the bacterial chromosome at a considerable distance from the structural gene, and it also seems to be dominant. One important difference between alkaline phosphatase and β -galactosidase is that in the former no period of constitutive synthesis is observed after the genes are transferred. If both of these systems are to be interpreted on the same model, one must conclude that the repressor for alkaline phosphatase can be made much more rapidly than that for β -galactosidase.

One question which immediately arises in connection with such repressor genes is whether the product of the gene is a protein. In further experiments with β -galactosidase, Pardee & Prestridge (154) showed that its formation is not blocked by the action of 5-methyl-tryptophan which interferes with protein synthesis. They suggested the possibility that the product of the repressor is an RNA moiety.

A new type of gene mutation, reported by Jacob & Monod (155), also affects the rate at which the structural gene makes protein; however, it differs from the mutations in the repressor gene in that it only affects the structural genes that are located on the same chromosome. A cell can be made diploid for the structural gene so that it is contained in two different chromosomes. If the normal gene is on one chromosome and a mutation of this gene, which makes an altered protein, is on the other, it is possible to determine which of the two is functioning. For the normal *i* gene both proteins were always controlled in the same way. However, for the new type of control mutation, designated "operator" (*o*) only that protein controlled by the structural gene

in the chromosome which contained the operator mutation was made in the absence of inducer. Another mutation which appeared to affect the operator gene resulted in the inability of the cell to make any β -galactosidase or any permease, the material responsible for concentrating galactosides within the cell. Jacob (156) interpreted these results as indicating that the operator gene was the site of action of the repressor. Equally, of course, one could think of the product of the operator gene as the site of action of the repressor. However, the operator mutation affected both β -galactosidase and the galactoside permease system, thus supporting the idea that the repressor acts at the level of the gene itself. According to this picture then, there exist, besides the structural genes which determine the amino acid sequence of the proteins that a cell can produce, two additional types of genes. One controls the formation of a repressor or a part of the repressor molecule which may be a polynucleotide. The second new type is the operator gene, which is thought of as a switch that responds to the attachment or detachment of the repressor molecule by turning off or turning on the structural genes adjacent to it. The operator-type mutation has so far only been observed in the β -galactosidase system.

Linkage of biochemically related genes.—Demerec and his associates (157) reported several years ago that in *Salmonella typhimurium* the genes which control enzymes in the same biochemical pathways are frequently closely linked on the chromosome. Their findings have been subsequently extended and confirmed for many enzyme systems of *Salmonella* and for other bacterial systems. [See Demerec & Hartman (158) for a complete review on this subject.] The linkage of the β -galactosidase structural gene and the galactoside permease gene mentioned above is another example of the same phenomenon.

In addition to the close linkage, it was also found that the order of the genes along the chromosome is frequently the same as the order of the biochemical steps in the biosynthetic pathway. A particularly complete study of such linkage was carried out (159, 160, 161) for seven genes which control the histidine biosynthetic pathway. The genes are linked in a region of the chromosome which is probably less than 1 per cent of the total bacterial chromosome.

An additional phenomenon which appears to be related to genetic linkage has been described by Ames & Garry (162) as co-ordinate repression. It was shown that four of the enzymes in the histidine pathway are all repressed by histidine and, furthermore, that when the histidine content of the cell or the degree of histidine starvation in the cell is varied the rate of synthesis of these four enzymes changes in an exactly parallel manner; thus, the ratio of the four enzymatic activities remains the same regardless of the degree of repression. This, of course, might imply that the enzyme molecules themselves are associated within the cell, and this possibility has certainly not been excluded. Jacob's (156) concept of the operator gene could also explain such co-ordinate repression, with the assumption that the entire genetic region could be turned on or off by the same operator gene.

In the fungi *Aspergillus* and *Neurospora*, linkage of genes for physiologically related enzymes has, in general, not been found, and in several instances an almost random distribution of genes has been observed for the same enzyme sequences which are linked in the enteric bacteria [see Pontecorvo (163) for review]. Gross & Fein (164) and Wagner *et al.* (165) have, however, found extremely close linkage in one case for enzymes involved in aromatic amino acid synthesis and in the other for valine-isoleucine synthesis.

Complementation.—When two different mutant genes, each of which is altered with respect to the same enzyme, are both contained in a single cell, some degree of enzymatic activity is occasionally restored without the intervention of genetic recombination. This phenomenon has been designated "complementation."

There seem to be two different ways in which this phenomenon is manifest. The first type occurs in tryptophan synthetase in *E. coli*, in human hemoglobin, and probably in the esterase of maize studied by Schwartz (166). In these cases, any mutation in the gene that controls one of the two separable parts of the protein will yield normal protein when combined with any mutation in the gene for the other part. Physiologically, these systems behave in the same way as those that affect different enzymes in the same biochemical pathway. The second type can be called intragenic complementation. Not all genes have shown this type of complementation, and, when it has been found, only a fraction of the mutations that affect a given gene show the phenomenon. Complementation maps, based entirely on the presence or absence of such complementation between pairs of mutants, can be constructed. A particular mutant is represented as a line on a complementation map, and the lines for two mutants overlap if the mutants do not complement each other and do not overlap if the mutants do complement. In all cases reported so far, the data could be represented on a linear complementation map. Case & Giles (167) reported an extensive series of complementation and genetic analyses on 75 pantothenic acid-requiring mutants of *Neurospora crassa* blocked at the step of the conversion of ketovaline to ketopantoic acid. From studies in other organisms, these mutants appear to involve a single enzyme, but the enzymology has not been worked out for this system. Of the 75 mutants, 23 show complementation, and there is no obvious clustering of this class on the genetic map. Although there is a rough correspondence between position on the genetic map and position on the complementation map, there are several clear exceptions to the colinearity of the two maps.

Fincham (168) found that the glutamic dehydrogenase of *Neurospora* made in a particular complementing pair, could be distinguished from the enzyme in the wild-type preparations in their thermal activation between 20° and 30°C. and their thermal stability at 60°C. It was suggested by Fincham (134) that intra-allelic complementation might result from the interactions of identical subunits necessary to form an intact enzyme molecule. It is supposed in this theory that two individually defective peptide chains could

provide mutual support and form a correctly folded protein through copolymerization. Crick & Orgel (169) have developed this type of theory in considerably more detail and have discussed both the limitations and general properties of such models. All models so far discussed, which are based on protein-protein interaction as the explanation of complementation, would predict that complementation maps should not necessarily be linear. Although no exception to linearity has so far been reported, the number of individual mutants examined for complementation is not large. On the other hand, any theory that is based on the interaction of identical subunits would be disproved by the finding of intragenic complementation for an enzyme that is known not to be made of polymerized subunits.

Woodward (170) has observed complementation *in vitro* for the adenylosuccinase enzyme; i.e., two different mutant strains, each of which lacks enzymatic activity, can be mixed in the cold after rupture of the cells and enzymatic activity recovered in the mixed extracts. This result seems to support the hypothesis that complementation is attributable to the protein interaction of the component parts of the enzyme.

In all cases studied, there is complete complementation if two mutations affect different enzymes and growth of the deficient organisms is taken as the indication of complete function (3, 160, 171).

Infectious and transforming nucleic acids.—That free nucleic acid can carry genetic markers from one bacterium to another (transforming principle) and can initiate viral infection (e.g., TMV-RNA) has been well-established and previously reviewed (172). In regard to infectious RNA extracted from plant viruses, recent experiments have demonstrated the phenomenon in a number of new viral-host systems (173, 174, 175). Although in most cases the degree to which the RNA has been freed of protein is still in doubt, it has been shown that the course of the infection is more rapid with free RNA than with intact virus (176). Infection with purified RNA from poliovirus has also been demonstrated (177).

Infectious DNA from polyoma virus was obtained by phenol extraction [DiMayorca *et al.* (178)]; no detailed report was given on the purity of the material obtained. It was sensitive to DNase and behaved chemically like free DNA. The urea-disrupted phage, studied by Fraser and co-workers (179) was resistant to DNase and infectious on bacterial protoplasts, but not on intact bacteria. No infectivity has been obtained with phage-T2 DNA extracted by phenol or released by osmotic shock, and it now seems likely that the urea-disrupted material is partially damaged phage which still retains much of the phage protein and is probably a more or less intact virus particle with some damage in its tail structure (180, 181). Hogness & Kaiser (182) have demonstrated transformation of *E. coli* by DNA isolated by phenol extraction from defective λ phage particles. The material has been shown to be free DNA by its sensitivity to DNase, its heat resistance, and by its buoyant density. The transformation occurs only if the recipient cell is simultaneously infected with an ordinary λ phage. Since the transformants carry phage ge-

netic markers from the extreme ends of the known genetic map of λ , it is likely that a single piece of DNA carries the entire genome of the phage.

The process of bacterial transformation has been studied by Fox & Hotchkiss (183) who used P^{32} -labeled DNA which was allowed to interact with the recipient cell for only a short time. They found that DNA reisolated from the transformed cells had the transforming activity, per unit of P^{32} , equal to that of the original labeled DNA. Furthermore, in the newly transformed cells the added marker multiplied at the same rate as a genetic marker of the recipient cell itself. When closely linked genetic markers were used, one in the transforming DNA and one in the recipient cell, Fox (184) showed that the DNA re-extracted from the transformed cells showed genetic linkage of the two markers after a short time, and the DNA synthesis did not seem to be required to establish this linkage. Immediately after transforming principle is added to a cell, the attached material is found to be resistant to external DNase, but is not recoverable as a biologically active transforming principle. This eclipse period terminates in about 10 min., and at this time the linkage with the recipient genes is already established.

Episomes.—A new type of genetic interaction, which may be of great general importance, has been elucidated by Jacob & Wollman (185, 186). In bacteria, it is possible to transfer genetic material by phage-mediated transduction, by transformation with free DNA, and by oriented chromosomal transfer in direct cell contact. In each of these cases the added material is either integrated into the linkage group of the recipient bacterium or it remains in the cytoplasm for a short time before being lost by dilution, as the cells divide. However, a new type of genetic entity which can interact with the cell in a variety of ways has been found in *E. coli*. These factors, called episomes, can be integrated into the bacterial chromosome, or they can exist in the cell as non-chromosomal self-duplicating entities which endow the cell with certain heritable properties. In addition, the cell may lack the episomal factor entirely. If a cell does not have the factor, it can be infected either by cell contact or by a virus carrying the episome. If, on the other hand, the episomal factor is integrated into the bacterial chromosome, the cell is rendered immune to infection from the outside, and multiplication of the factor in a non-integrated state is suppressed.

Genetic entities with these properties were first observed in a case of the lysogenic virus, which can exist in all three episomal states: as a free virus, as the vegetative virus growing in the cell cytoplasm or integrated into the bacterial chromosome, and as a provirus of the lysogenic cell. The cell without the virus is sensitive to infection, but the lysogenic cell becomes immune. The colicinogenic factor responsible for the production of a specific bacteriostatic agent of high molecular weight has the same three episomal states (187) as does the fertility factor which determines the mating behavior of the bacteria (186). A clear-cut verification of the episome concept was provided by the finding that the fertility factor can carry with it a piece of the bacterial chromosome when it makes the transition from the integrated to the

non-integrated state [Jacob & Adelberg (188); Adelberg & Burns (189)]. When the cell carrying this type of F-factor comes in contact with an F⁻ cell lacking the factor, the bacterial genes are transferred with very high efficiency along with the F-factor. In this way, cells can be prepared which have several copies of the same gene, one integrated in the bacterial chromosome and the others multiplying in the cell as extra chromosomal genes. The structural genes that control the enzyme β -galactosidase and also alkaline phosphatase, as well as repressor genes for each of these enzymes, have been transferred from cell to cell by means of attachment to the F-factor (153, 186).

Nothing is known of the chemical nature of this episome, except in the case of the lysogenic virus. When the factor is carried by the free virus, it has been shown to be DNA (182). Since the evidence from bacterial transformation (190) and from chromosomal transfer (29) suggests that DNA carries the genetic information in these organisms, it seems likely that the episome is, or at least contains, DNA in all its states. Hirota & Iijima (191) and Hirota (192) have studied the elimination of the F-factor from its non-integrated state by the action of the acridine dyes, acroflavine and acridine orange. These agents are active on the non-integrated F-factor but do not affect it when it is integrated in the bacterial chromosome.

MUTAGENESIS

Chemical mutagenesis.—A large number of base analogues are known to induce mutations, and, while many, but not all, of these analogues have been shown to be incorporated into DNA, it is usually assumed that the mutagenic action is related to such incorporation. It has been suggested that analogues act by causing a mistake in the base-pairing of the DNA with the result that a "transition" occurs, which changes the guanine-cytosine base pair to adenine-thymine or vice versa [Freese (193); Litman & Pardee (194)]. Since bromouracil can replace thymine almost completely in the T phages and still leave most of the particles viable (194), and since transforming principle heavily substituted with bromouracil is still active (195), it is clear that incorporation itself is not the only requirement for mutagenesis. Freese postulated that mutations arise from more frequent tautomeric shifts which might occur with the analogues and lead to mistakes in base-pairing. The pairing mistakes could occur either during the incorporation of the analogue or by a tautomeric shift in the already incorporated base, which would allow incorrect pairing to occur on a subsequent replication. In this way, an analogue could cause either of the following pairings: adenine-thymine \rightarrow guanine-cytosine or guanine-cytosine \rightarrow adenine-thymine. Rudner reported experiments to test these theories (196). Experimental support for this theory of mutagenesis comes from the fact that mutations induced by base analogues can be reversed by such analogues, but these substances, in general, cannot induce spontaneous mutations to revert (193).

In addition, Demerec (197) reported that spontaneous mutations and

ultraviolet-induced mutations led to what were apparently deletions in the *Salmonella* chromosome, whereas bromouracil induced only point mutations.

The chemical nature of the mutagenic event can be directly studied by the use of reagents like nitrous acid (198) and ethyl methyl sulphonate (199), both of which cause mutations when reacted with nucleic acids in extracellular virus; this is in contrast to the base analogues, which act only on systems with active nucleic acid metabolism. Mundry showed that nitrous acid is a true mutagen and its action on TMV is not that of selecting pre-existing mutants (200). When nitrous acid reacts with TMV-RNA, the deamination of virtually any base produces some biologically observable effect (201, 202). Poliovirus (203), another RNA virus, and transforming DNA (204) are also sensitive to the mutagenic action of nitrous acid.

An elegant study of the mutagenic action of nitrous acid was carried out on DNA and the T2 phage. Schuster (205) studied the ratio of the deamination rates of adenine, guanine, and cytosine at different pH values. Vielmetter & Schuster (206) used the change in deamination rate at pH 4.2 and pH 5.0 to elucidate the action of nitrous acid in producing mutations and inactivating the phage T2. The rate of production of r_{II} mutants was found to decrease by a factor of 88 when the nitrous acid was applied at pH 5.0 rather than at 4.2; this is the same decrease which was observed in the deamination rate of both adenine and cytosine in the T2-DNA. It was therefore concluded that deamination of adenine or cytosine, or both, but not the deamination of guanine, was responsible for mutations. It was pointed out that this was to be expected from the base-pairing scheme of Watson & Crick (21), since the amino group of guanine is in the 2' position and is not involved in the base-pairing. On the other hand, the ratio of inactivation at pH 4.2/pH 5.0 was approximately 30, which is the same as the corresponding ratio for the deamination of guanine. Therefore, it was suggested that the bulk of the inactivations is attributable to the effect on guanine. At either pH 4.2 or 5.0, transitions induced in any one of approximately 370 base pairs were found to lead to visible r_{II} mutations. This is approximately one-half to one-third of the total base pairs in the r_{II} region, as estimated by Benzer (207).

Tessman (208), by use of nitrous acid treatment of free phage T4, and Pratt & Stent (209), by use of 5-bromouracil on phage growing in infected bacteria, found that heterozygous mutants were produced. These particles gave rise to mixed clones of mutant and non-mutant progeny on further growth in normal cells. Since no phage is observed to be heterozygous for the complete genome, it has been concluded that the phage contains only a single genetic linkage group. These mutational heterozygotes were interpreted as an alteration in one of the double strands of a normal DNA molecule. In contrast, it was shown that nitrous acid treatment of the phage ϕX 174, which seems from physical-chemical evidence to contain single-stranded DNA (119), produces mutants but not heterozygotes.

Radiation damage to DNA.—Of the many reports of the effects of ionizing radiation on organisms and nucleic acids, only those will be discussed in

which direct attack on the genetic material can be construed. Radiation in aqueous systems can produce a number of reactive substances (210, 211), and the subsequent reactions in living cells have yet to be elucidated. Studies on radiation and carcinogenesis have been recently discussed elsewhere (212, 213).

Benkers & Berends (214) irradiated a frozen solution of thymine and detected the formation of a dimer. Studies on the nucleic acids have shown that radiation causes chain scission which is sometimes accompanied by aggregation (215 to 220). Hems (221) irradiated DNA with electrons and found that sugar and base destruction and chain scission occurred. In several cases, an increase in degradation occurred in the presence of oxygen.

A similar oxygen effect has been observed in the "suicide" of *E. coli* from the decay of P^{32} in DNA (222). Matheson & Thomas (223) found that the presence of a radical-quenching reagent also lowered the death rate of phage on the decay of incorporated P^{32} . These observations suggest that, if the killing results from a single decay which ruptures both strands of DNA at the same time [see Stent & Fuerst (224)], at least one of these breaks is produced by a chemical rather than a physical reaction. The reaction is probably brought about by a free radical of very short lifetime and range, since the same activity of P^{32} in the solution external to the organisms has little killing effect.

Hutchinson & Arena (225) showed that the inactivation of transforming principle by 1 Mev. electrons increased on wetting the DNA. They attributed the enhanced damage to the action of short-lived radicals, which, they calculated, were effective if they were produced within a 10 Å shell around the DNA. Ellison & Beiser (226) have studied the inactivation of transforming DNA by ultraviolet irradiation. Krieg (227) has demonstrated that ultraviolet irradiation of extracellular phage can produce mutations.

A number of papers have confirmed the earlier work of Zamenhof and others on the increased ultraviolet sensitivity of DNA containing 5-bromouracil. Greer has shown that the sensitivity probably reflects a decrease in the stability of the DNA rather than any disturbance to the metabolism of the parent cell (228). More recent work also shows that phage heavily labeled with bromouracil becomes sensitive to visible light (229).

Englander *et al.* (230) measured the inactivation of TMV under x-rays and attributed the loss of infection to the rupture of the RNA strand. Emerson *et al.* (231) compared the radiation sensitivity of DNA and nucleoprotein and concluded that the protein protects the DNA. They suggest that the variation of the sensitivity of the cell to radiation at stages of the mitotic cycle might relate to the integrity of the protective protein sheath. Norman (232, 233) has reported the heat and ultraviolet inactivation of polio virus RNA.

Freifelder & Uretz (234) reported that visible-light irradiation of acridine-orange treated cells produced mutagenesis and killing in yeast and *E. coli*. Acridine orange is preferentially bound to DNA, and it appears that the damage to the cell is through RNA or DNA, and most probably the latter.

Photoinactivation studies on phage treated with the related dye, proflavin, (235) probably reflect a similar dye-mediated damage to DNA.

Chromosome structure.—Kellenberger has recently summarized the results of electron microscopic studies on the bacterial nucleus. Steffensen (236) and the discussers of his paper have reviewed similar studies on chromosomes [see also (237, 238)]. In each case, fibrils have been observed with a diameter close to that attributed by Wilkins *et al.* (239) to nucleohistone. In the case of bacteria, it is not unreasonable to assume that the fibril observed is the genome, of which DNA forms at least a major fraction. In chromosomes, basic proteins and others occur as well as RNA and DNA, but the manner in which these components are integrated remains unknown.

Steffensen & Bergeron (240) and Hagen (241) have commented on the importance of calcium to chromosome structure. Brachet (242) has discussed the role of RNA in the nucleus, and Taylor (243, 244), using tritiated thymidine for autoradiography, has reported and reviewed further studies on the replication of chromosomes. These studies all relate to a gross description of the chromosome—whether a single highly convoluted sequence of DNA molecules (with or without “linkers”) or a number of parallel fibers carry the genetic information remains to be seen.

Studies on the histones attached to DNA in animal cells have shown that the basic proteins increase in step with DNA through the mitotic cycle (245, 246) and are conserved through mitosis (247). The presence of proteolytic enzymes in preparation of these basic proteins (248, 249, 250) has made some of the earlier histone investigations of doubtful value, but it is certain that the histones are a mixture of different proteins (251, 252). Bakay *et al.* (253) have fractionated nucleoprotein on an ion-exchange column, but it remains to be demonstrated if the parallel fractionation of nucleic acid and protein is real or if the components have separated and recombined to produce artifacts.

The function of the basic proteins remains obscure. Ames & Dubin (254) have shown that, in bacteriophage, DNA can be combined non-specifically, even in the intact virus, by metals or polyamines; it is possible that the histones similarly serve to balance the charge on the DNA. If this is the case, however, the curious transition from histone to protamine [for example, see (255)] remains to be explained. Mauritzen & Stedman (256) have given further evidence to supplement an earlier report that at least some histones are tissue specific. As these authors suggest, this finding may indicate that, in some way, the histones implement cell differentiation. Kit (257, 258) has confirmed Kondo & Osawa's report (259) that different tissues, even malignant cells of the same species, yield DNA with the same chromatographic profile and composition.

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BIOCHEMISTRY OF THE DIVIDING CELL^{1,2}

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The normal form of cell reproduction involves the duplication of the nuclear genetic material, the partitioning of the genetic material into two equivalent, or "sister," nuclei, and the partitioning of the cytoplasm into two separate cells, each with one of the nuclei. Nuclear division is not necessarily followed by cytoplasmic division; the production of multinucleated cells is a common occurrence in nature and in experiments. On the other hand, cell division without nuclear division, which would produce anucleate cytoplasmic masses, is a rare occurrence, obtainable only under special experimental conditions (36, 70, 159).

In all plant and animal cells, in protozoa, and in a great many algae and fungi, cell division involves the method of mitosis. The defining features of the mitotic method are: the "packaging" of the genetic units in chromosomes, the condensation of the chromosomes at the time of division, and the transportation of sister chromosomes to opposite poles. Thus, the major problems of mitosis are problems of structure and of very specific movement; therefore, a discussion of the biochemistry of the dividing cell takes place in a different context from discussions of metabolic biochemistry and biosynthesis. It involves the polarization of the whole cell, specific "connections" or "attractions" between large bodies, large-scale movements, and, normally, an exact partitioning of the entire cell.

Unfortunately, we do not have an adequate account of the method of nuclear division in bacteria and blue-green algae in the sense that mitosis is the method employed by plant and animal cells. A review of the status of cytological knowledge of bacterial division is hardly in place here, but few are convinced that a chromosome cycle or a mitotic spindle has been observed (93). Since the chief business of bacteria is to make more bacteria, an understanding of the biochemistry of division of this group is important for its own sake. But we cannot assume, at present, that fundamental similarities exist between the dividing bacterial cell and other kinds of cells in division. Rather than exploit the unitarian doctrine that has been so valuable in solving other biological problems, we have to put it to a severe test here and be prepared for evidence of truly fundamental diversity. If, in the end, we must divide the biological world into forms that do and forms that do not employ mitosis, the distinction is hardly less fundamental than others that have been invoked in thinking about evolution.

This article is planned to call the attention of the readers of the *Annual*

¹ The survey of the literature pertaining to this review was concluded in November, 1960.

² The following abbreviations are used: ATPase for adenosine triphosphatase; TCA for trichloroacetic acid.

Review of Biochemistry to the central questions of the biochemistry of cell division and to the tentatively factual basis for formulating the questions. Statements that are presented as generalizations must also be regarded as expressions of the author's opinions; let the reader beware. The review is confined to direct studies of the dividing cell and the division apparatus and excludes one very large field of investigation: the study of mitotic stimulation and inhibition by hormones, carcinogens, antimitotic drugs, "mitogenetic rays," conventional irradiation, etc. Perhaps a clever reviewer who knows this vast literature might draw some important inferences concerning the biochemistry of division, but most of the investigations are designed for other legitimate purposes and are not addressed to the analysis of division itself. A comprehensive book (12), an international symposium (146), and some important reviews (e.g., 65, 67) deal with the effects of chemical agents on mitosis.

No attempt has been made in this review to give all the available references, and undoubtedly important work has been overlooked, either unintentionally or because it is peripheral to the questions chosen for discussion.

PREPARATIONS FOR DIVISION

The over-all reproductive cycle of cells dividing by mitosis is divided into two periods: (a) a period of preparations for division, corresponding roughly to the interphase period, or the period when the chromosomes appear dispersed and are surrounded by a nuclear membrane; and (b) the period of division itself, beginning when the chromosomes begin to condense. It is obvious, visually so, that the entrance of a cell into mitosis involves major transformations of its structure, and the problem is to define the two phases of the life cycle and the transitions from one to the other in molecular terms.

Students of the subject now formulate the problem in terms of the following hypothesis. During interphase the cell must complete a number of prerequisites to division. These may be viewed as parallel events which may interact normally in various ways but can be dissociated experimentally. The minimum condition for the entrance of the cell into division is the completion of the last prerequisite. This formulation, has been arrived at by a number of the workers in the field (63, 64, 76, 124, 137, 156). A diagrammatic representation of the division cycle in these terms is given by Mazia (78). Let us review the characteristics of the processes in the interphase cell which can be regarded as preparations for division.

Growth.—If we take as a norm for cell growth the doubling in amounts of all of the active and structural molecules of the cell, we can ask whether normal growth as such is a prerequisite to division. There is a great deal of evidence that it is not, that only certain categories of biosynthesis are strictly required as prerequisites. [For a review of growth-division relationships, see Swann (137, 138) and, more recently, Prescott (109)].

DNA synthesis and chromosome reproduction.—It now seems certain that the doubling of DNA in each growth-division cycle of cells dividing by mito-

sis takes place between divisions and never during division. A statement that DNA synthesis takes place somewhere between the telophase of one division and some time in the prophase of the next covers all the cases studied. In any one cell type, the period of DNA synthesis is rather characteristic; in mammalian cells it generally occurs during the latter part of interphase, but in other kinds of cells it may come quite soon after division or may be spread over most of the interphase. The literature is voluminous, but only a few sources need be cited (16, 22, 140). Quantitative cytochemical studies have also shown that the increase in nuclear histones takes place between divisions, paralleling the increase in DNA. Thus, if DNA and histone are regarded as the chief molecular components of the chromosome, we may say that the division process itself effects the distribution of genetic material that has been synthesized before division.

The total reproduction of the chromosome may involve the synthesis of other chemical components and the production of "non-genetic" structures such as centromeres (kinetochores), which are involved in the mitotic movements. It has been argued (81) that these poorly understood additional events may have important implications for our picture of the biochemistry of division.

If the reproductive cycle of bacteria involved a mitotic process, it might be expected that DNA synthesis would be phased as it is in cells of higher organisms and would be confined to an interphase period. Such a state of affairs has been reported by workers who have used artificially synchronized populations (16a, 62, 73, 128). But other studies on bacteria growing under normal conditions give strong evidence that DNA synthesis is a continuous process, not interrupted during the period of division or at any other time (123, 152). Since there is so much evidence that chromosomes in the "condensed" mitotic condition do not synthesize DNA (18, 101, 144, 145); the observations on normally growing bacteria suggest that their chromosomes may not go through a mitotic cycle at all.

Prior reproduction of the chromosomes is generally a condition of cell division. Apart from the very important case of meiosis, few cases have been observed in which cell division results in a reduced DNA content. A division of this kind following treatment of ascites tumors with 5-fluorouracil has been reported (68).

Reproduction of the centrioles.—In animal cells at least, the mitotic apparatus is polarized by definite particles called centrioles. Functionally, the centrioles define the destinations of the chromosomes in mitosis. Their structure is being studied by electron microscopy (e.g., 3, 10, 11, 107), and a characteristic cylindrical body about 2000 Å and 250 to 350 Å in diameter composed of a cylindrical array of about nine groups of tubular-appearing elements is found in many kinds of cells. The reproductive history of the centrioles has been described by Mazia, Harris & Bibring (83), who confirm the classical observation that the reproduction of the centrioles for a given division takes place toward the end of the previous division. Since they are most

important in cell division, as well as being a classical example of an extranuclear self-reproducing body, it is very important to explain their chemistry. Very little is known. Staining reactions indicative of RNA have been reported (2, 133), but such tests cannot be obtained on all kinds of material. It would be most important to establish unequivocally the presence of a nucleic acid component, as well as to work out the molecular basis of the very characteristic structure of the centrioles. Their isolation will pose considerable difficulties, for they are small and number only two to four in a typical animal cell.

Provision of molecular components of the mitotic apparatus.—The mitotic apparatus—the ensemble of structures involved in the movement of the chromosomes, including the spindle and aster—often occupies a considerable fraction of the volume of the dividing cell; this is a matter of simple observation. Evidence from interference microscopy (69, 88) demonstrates that its density is no less than that of the rest of the cytoplasm. Analysis of the isolated mitotic apparatus in the case of the sea urchin egg (85) shows that it accounts for about 10 per cent of the protein content of this cell during the division period. The spindle and, in many animal cells, the asters account for the largest part of the bulk of the mitotic apparatus. Even allowing for the fact that these "fibrous" components are embedded in a matrix of cytoplasmic ground substance, which includes particles of ribosomal dimensions (33, 106) and may contain some endoplasmic reticulum (108, 119), we may suppose that a considerable amount of material, proteins being the major component, is involved in the characteristically oriented fibrous structure. Is this synthesized at the time of division or is it assembled from molecular components the synthesis of which may be regarded as a necessary preparation for division? Present evidence supports the latter conclusion. Immunochemical studies (150) on the sea urchin egg have demonstrated that the antigens characteristic of the mitotic apparatus are present before the apparatus is organized. Treatment of new fibroblasts with chloramphenicol some time before division inhibits the formation of a mitotic apparatus, but the "point of no return" is passed before the apparatus actually appears; that is, chloramphenicol is ineffective at the time the apparatus is forming (143). An electrophoretic study of proteins extracted from lily anthers shows the disappearance of a major component during the time the cells are in division, and a possible interpretation is that this component is associated with the mitotic apparatus and becomes insoluble during division (94). The tentative conclusion is that the substances of the apparatus must be synthesized before the onset of division.

The "energy reservoir."—The energetics of the dividing cell will be discussed in a later section. Suffice it to point out here that withdrawal of energy-yielding substrates (19, 20) or introduction of inhibitors of oxidations and phosphorylations (CN^- , CO, azide, DNP, etc.) will prevent division if applied early enough, but there is a "point of no return" just before division or in prophase. The most successful general interpretation of these observations,

even allowing for criticisms of specific hypotheses (32), employs the hypothesis of an "energy reservoir" (136, 137). In the most general terms, this says that the energetic price of the division process itself is paid before division, whether in the form of the storage of specific high-energy compounds (136, 137) or of the production of the mitotic apparatus in an activated condition (80). Thus, the mobilization of energy for division is a preparation and prerequisite to division.

Conclusions.—The number of essential events in the reproductive cycle of cells (with the possible exception of bacteria) which take place before manifest division and are to be regarded as preparations for division is impressive. It includes all of the genuine reproductive events at the molecular level (replication of the genetic material and of the chromosomes), the major macromolecular syntheses (provision of spindle substance), and the fundamental energy-mobilizing steps. Surely other essential preparations will be covered. For example, there is evidence in some cases of an active synthesis of RNA, especially nuclear RNA, just before division (91). It would appear that understanding the problems of the biochemistry of division itself have to do with structural assembly, orientation, and movement more than with biosynthesis.

THE CHROMOSOME CYCLE

The entrance of the cell into mitosis and its passage through the various phases is recognized by microscopic observation of the coiling cycle of the chromosomes. Our knowledge of the cytological aspects at the level of the optical microscope is voluminous, and we may expect major advances in the electron microscopical analysis. [For samples of current views on the electron microscopic image see (53, 90, 96, 114)].

The biochemical problems, as we can formulate them now, are: (a) the causation and molecular character of the coiling or "condensation" of the chromosomes and (b) the changes in the chemical composition of chromosomes during mitosis.

Very little experimental work has been done on the coiling mechanism. The problem has been analyzed in a thoughtful way by Anderson (4), who proposes an unusual model of coiling. His interesting suggestion is that organic polycations may be a causal agent in chromosome condensation, and this finds some support in observations of the effects of various artificial and natural polyamines on the state of chromosomes in isolated nuclei (6).

The composition of chromosomes in the course of the mitotic cycle has been investigated by cytochemical methods. Thus far, we lack a satisfactory method for the mass isolation of chromosomes at various stages of mitosis. This problem will undoubtedly be solved; in part its solution will depend on the perfection of methods for synchronizing division in cell populations. Such methods are discussed in a number of articles (see 98, 156).

The cytochemical evidence reveals that there are important changes in the chemistry of the chromosomes as they pass through the mitotic cycle.

A study with the interference microscope (112) suggests a decrease in mass during the period of coiling (condensation). There is as yet no exact chemical information on the nature of the indicated losses. They may reflect, in part, the loss of nucleolar substance. It has been found recently that the nucleolus may be characterized cytochemically by the presence of substances of unknown chemical composition which are recognized by their ability to reduce silver under specified conditions (25, 142). This material is also associated with the chromosome arms during early prophase. It is not seen in the cytoplasm during interphase. As prophase proceeds, it is lost from both the chromosome arms and the compact nucleolus at the time the latter breaks down and is then detected in the cytoplasm. At the end of anaphase, it disappears from the cytoplasm and reappears on the chromosomes and in the nucleolus. This "cycle" could be of extraordinary importance, but cannot be evaluated until the silver-reducing component has been identified.

On the other hand, sensitive qualitative staining methods show certain additions to the chromosomes. Their RNA content increases considerably during the period of prophase coiling, as was first shown by Kaufmann *et al.* (54) and confirmed by a number of others working with different kinds of cells (15, 50, 71, 115). In effect, the chromosomes acquire an RNA component during prophase, carry it through metaphase and anaphase, and release it during late anaphase or telophase. A similar cycle has been observed (60) for a chromosomal component stained with orange G, which LaCour & Chayen have identified as phospholipid.

It is interesting to speculate on the significance of the addition of material to the chromosomes during division. At least three guesses, which cannot be dignified as hypotheses, are possible. First, the additions may be related to the process of coiling itself. Second, they may represent chromosomal components concerned with the mitotic movements but not with primary genetic functions. Third, we may be observing a device for a directed distribution of non-genetic components of the cell by the rather precise mitotic mechanism. There is a speculative possibility that this could be related to the causation of differentiation.

In the reviewer's opinion, cytochemistry has provided a solid, if thin, foundation for a more detailed chemical study of chromosomes in mitosis, and the limiting factor is our lack of adequate methods of mass isolation.

THE MITOTIC APPARATUS

Some progress has been made toward a chemical description of the mitotic apparatus. The results so far have been useful in formulating hypotheses concerning its structure and assembly. But little progress has been made toward revealing how the chromosomes are moved, which, after all, is the ultimate goal of the study of the chemistry of mitosis. Our present picture combines results obtained by cytochemical methods, by studies with polarized-light, and by direct chemical studies of the isolated mitotic apparatus.

Isolation techniques.—Most of the work on the isolated mitotic apparatus has been carried out on eggs, especially sea urchin eggs. So far, this has been the only material providing large numbers of synchronously dividing cells. The chief difficulty in developing an isolation method results from the extreme instability of the mitotic apparatus. It is not preserved in the homogenization media that have been favored for the isolation of mitochondria, nuclei, etc., and all of the existing techniques call for stabilizing reagents which may be a source of biochemical artifact.

The original isolation technique of Mazia & Dan (82) involved: (a) arrest and preservation of the dividing eggs in 30 per cent ethanol at -10° , which contributed to the stabilization of the mitotic apparatus; (b) further stabilization with H_2O_2 ; (c) solubilization of the cytoplasm with Duponol (sodium dodecyl sulfate), thus liberating the mitotic apparatus; and (d) purification by centrifugation. This passed through various modifications (26, 74, 75, 77) involving elimination of the peroxide and the use of gentler dispersing agents. The technique used for most of the chemical studies called for stabilization by prolonged immersion of the cells in the cold 30 per cent ethanol followed by the use of digitonin as a dispersing agent (75). Attempts to eliminate the alcohol and the detergent, both of which might account for early failures to recover enzyme activities in the material, led to a more recent method in which the mitotic apparatus is isolated in a sucrose medium with dithiodiglycol as the stabilizing agent (79). It cannot be claimed that the mitotic apparatus has as yet been isolated in a truly "native" form until it can be made to perform some of its mitotic functions *in vitro*; at present, each isolation method is judged by its usefulness for a particular analytical investigation.

Descriptive chemistry.—The mitotic apparatus isolated by the alcohol-digitonin method has been characterized in a number of ways. It consists largely of protein, but contains 6 per cent, and perhaps more, RNA (158). The nucleotide component appears to be RNA of about the same average base composition as the RNA of the whole cell (158). An earlier report that it was chiefly composed of adenylic nucleotides (77) was the product of inadequate analytical technique. This RNA-protein composition has also been deduced from studies of the mitotic apparatus of the Cyclops eggs by the x-ray absorption technique of measuring mass before and after treatment with ribonuclease (134). The possible presence of a lipid component has not been investigated properly; the results would have been rather meaningless following isolation in digitonin. The question of lipids in the mitotic apparatus has become urgent in view of electron-microscopic evidence that the "fibers" seen by light microscopy may consist in part of tubular filaments, which are possibly related to the endoplasmic reticulum (10, 106).

The protein component has been analyzed in some detail. The protein obtained from the dissolved mitotic apparatus consists of two components when studied by electrophoresis or analytical ultracentrifugation (75, 158). One component predominates. The average molecular weight is 315,000.

The isoelectric point is *ca.* 4.5. An amino acid analysis (75, 117) yields a profile strongly reminiscent of that of muscle actin. Thus far, immunochemical analysis reveals two major antigens (150). Unfortunately, all of the data are still limited to the mitotic apparatus from one source (sea urchin egg) and to preparations isolated by the alcohol-digtonin method.

Several studies employing staining methods for protein-SH (56, 57) show strikingly that the proteins of the mitotic apparatus are higher in —SH during certain phases of mitosis than those in other regions of the cell. There is evidence from staining reactions that the mitotic apparatus includes a polysaccharide component (46, 132).

As has been mentioned, a study of possible lipid components is badly needed in view of the electron-microscopic evidence for membranous and tubular structure in the mitotic apparatus.

Enzymes.—The most attractive enzymes to seek in the mitotic apparatus are those disposing of ATP; their presence would be called for by generalized theories of cellular motility (41, 149). No ATPase activity nor evidence of ATP-binding could be obtained with the isolated mitotic apparatus as long as isolation methods employing alcohol and detergents were used. With the introduction of the newer method cited above, an ATPase was found to be associated with the apparatus [data of R. M. Iverson cited by Mazia (78)]. A more detailed study (Chaffee & Mazia, in preparation) indicates that the fibrous components of the apparatus carry an enzyme that splits ATP, but not guanosine triphosphate, cytidine triphosphate, uridine triphosphate, adenosine diphosphate, or substrates for phosphomonomerases. It is a Mg^{++} -dependent enzyme with its pH optimum at 8.4. There is also ATPase activity in the particles "trapped" in the mitotic apparatus and set free when it is dissolved.

A cytochemical study of mitotic apparatus isolated from grasshopper spermatocytes by the alcohol-peroxide-Duponol method (104) gave positive results for alkaline phosphatase.

Intermolecular bonding.—Such a large and regular assembly of macromolecules as the mitotic apparatus calls for a consideration of the intermolecular bonds by which it is held together. Earlier work (74, 75) stressed the possible role of S—S bonds. The results of more exacting tests of this hypothesis have not given strong support to the hypothesis of S—S bonding, but persistently call attention to the participation of sulfur-bonds in the structure of the apparatus (56, 79). The best one can say at present is that there is a strong suspicion that some kind of bonding through S is important in the structure of the mitotic apparatus, and a speculative hypothesis stressing a more dynamic view of sulfur-bonding has been presented (81).

Studies of the effects of D_2O on mitosis (34) provide evidence that hydrogen bonding may play an important part in the structure of the apparatus. The possible role of polyelectrolytes has also been discussed. The pioneering studies of Heilbrunn (39) on the role of Ca in gelation reactions in general and in the formation of the mitotic apparatus in particular have been

extended by Anderson and collaborators (4, 5), who stress the possible role of polyelectrolytes in structure formation.

The stability of the mitotic apparatus is highly dynamic. In nature, it appears and disappears at appropriate phases of the cell cycle. Its bonding is sensitive to high hydrostatic pressure (102, 103) and to colchicine and related drugs. By application of isolation techniques, it has been found that the action of colchicine-like drugs is probably exerted at the level of intermolecular bonding, but that it is an indirect action, mediated by processes in the intact cell (122). A particularly informative approach to the dynamic stability of the mitotic apparatus is given by polarization-microscopic studies (48). It is observed that the birefringence of the mitotic apparatus increases with increasing temperature. Thermodynamic calculations from these measurements are consistent with the hypothesis that molecular orientation in the mitotic apparatus is the expression of a temperature-sensitive equilibrium between oriented and disoriented phases of a molecular population. In a sense, the dynamic character of the structure of the apparatus may be described by saying that its stability and orientation express a continuing activity of the whole cell.

ENERGETICS OF MITOSIS

The most dramatic expression of the energetic cost of mitosis is the movement of the chromosomes. In animal cells the constriction of the surface in cytoplasmic division is perhaps even more obvious. Because these events are most consonant with our kinesthetic prejudices as to the nature of work, we tend to anticipate that mitosis will be energetically expensive and to look for substantial increments of energetic metabolism at the time of division.

Respiration.—The most obvious measurement to test this anticipation is a measurement of cell respiration. The search for a change in the respiratory rate during division began a long time ago (summarized in 17) and has been pursued as methods have improved. It has long been clear that a sensational fluctuation was not to be expected. Careful studies on favorable plant material, lily anthers, have indicated that cell respiration might decrease during division (30, 131). Utilizing highly sensitive "diver" techniques, Zeuthen (154, 155, 157) has produced a long series of studies on various kinds of cells, especially eggs, which shows that there is a rhythm of respiratory rate associated with the cell cycle and that the period of mitosis is one of slightly depressed respiration (42). In no case was the amplitude of the changes very marked. A comparable series of studies employing a very sensitive "reference diver" system has been published by Scholander and collaborators (125, 126). They question the conclusion that the small rhythmic fluctuations in respiration are a consistent accompaniment of the division cycle, though some of their data show it. The controversial aspects of these differing conclusions have been discussed by Zeuthen (157). The only difference of opinion is between the view that there is no respiratory change associated with division and the view that there is a depression of the rate. There is no evi-

dence that the cell is panting very hard when it is dividing! Two kinds of conclusions are possible: either the immediate energy cost of division is no higher than the cost of the biosyntheses in preparation for division or the cost is paid in advance by the filling of an "energy reservoir."

Mitochondria.—Mitochondrial behavior during division is of interest in relation to the energetic problem and to the problem of the distribution of these bodies between the daughter cells. In some kinds of cells, particularly among insects, mitochondria assemble into bundles of long filaments which align themselves along the mitotic spindle. When the division furrow cuts through the spindle axis, these mitochondrial chains are more or less equally distributed between the daughter cells. Other forms of "chondriokinesis" are described in the older literature. In insect spermatocytes, mitochondria tend to accumulate near the surface of the nucleus during prophase, and it has been suggested that they play an active enzymatic role in the breakdown of the nuclear membrane (9).

In vertebrate cells in tissue culture, mitochondria tend to fragment during the mitotic period (23). In sea urchin eggs, the count of microscopically visible mitochondria declines sharply during mitosis and returns to normal between divisions; Agrell (1), who reports this behavior, correlates it with the decline in respiration.

These various observations cannot be correlated convincingly with the energetics of division, but the rapidly growing interest in structure-function relationships in mitochondria may justify their inclusion in a biochemical review.

Energy sources and the energy reservoir.—The basic observation is that it is impossible, once the cell has entered division, to interrupt that division by withdrawal of nutritional energy sources or by inhibition of respiration, glycolysis, or oxidative phosphorylation. One interpretation, by Bullough (19, 20), is that the cell, in order to enter division, has to pass through a pre-division phase, or "antephase," and that this passage has an energy requirement calling for glucose and for oxygen. For example, data on the stimulation of division by experimental wounding of tissue have been interpreted in terms of an enhanced diffusion of these metabolites. The idea of an antephase has been criticized in some detail (32), but the criticisms do not apply to the more general concept of an energy reservoir.

According to this concept, as developed by Swann (136, 137), the energy requirements for division itself are met by the accumulation of high-energy reserves before division, and it is further proposed that the precipitation of division follows the filling of the reservoir. (A mechanical model would be a siphon.) In a still more general form (80), it can be hypothesized that all the conditions of activation of the "working" molecules in cell division are met before division; this could imply either a conventional pool of high-energy compounds or the activation of the working molecules themselves. (The latter would be conceivable in mitosis, although not in muscular contraction, because the mitotic apparatus only works once; it then breaks

down and new ones are formed in the next division.) The best designed experiments on the "energy reservoir" are those of Swann (136), in which cells are maintained in an atmosphere of CO and the inhibition is switched on and off by means of light. If the inhibition is applied before prophase, division is delayed for a length of time exactly equal to the duration of the inhibition. If it is applied during a division, it has no effect on that division, but the next one is delayed by an interval corresponding to the duration of CO action. The effect of CO is not unique, but it can be controlled much better than that of other inhibitors.

This enlightening hypothesis has stimulated a search for the reservoir. In sea urchin eggs, on which so much of the work has been done, no build-up of ATP has been detected, and attention has turned to other high-energy sources, such as thiol esters (137). In the ciliate *Tetrahymena*, however, a considerable build-up of ATP and guanosine triphosphate before division and a breakdown during division have been established (105). Another type of strategy consists of attempts to relieve the inhibition of division by ATP. Marginal effects have been reported in the case of eggs of the annelid *Chaetopterus* (9a). The problem is complicated by the questionable permeability of the cell to ATP, but this is an interesting experimental design.

In a number of cases, such as sea urchin eggs (59) and mammalian epidermis (19, 20), the evidence that the energy requirements for division are met exclusively by aerobic pathways is unequivocal. On the other hand, there are numerous cases in which division proceeds under anerobic conditions; for example, it has been stated (44, p. 189) that the division of embryonic cells is relatively indifferent to anerobiosis.

METABOLIC STATUS OF THE DIVIDING CELL

Biosynthesis.—It has been pointed out that the major biosyntheses associated with mitosis take place before mitosis begins. The structural changes involved in division are so drastic that we may ask whether other biosyntheses continue during the division period. Obviously, the first question is whether the cell continues to grow in mass while it is dividing. In some cases the answer is negative. Measurements of the mass of *Amoeba proteus* (110) suggest an interruption of growth before and during division. In the case of *Paramecium* (58), cell mass may be reduced at division. A reduction in mass of HeLa cells during division is considered possible by Sandritter *et al.* (121). In the fission yeast, *Schizosaccharomyces pombe* (86), total dry mass, measured by interference microscopy, continues to increase during the division period, whereas cell volume remains constant. A similar situation is found in budding yeast (87). Clearly then, an interruption of over-all biosynthesis measured as mass can be an accompaniment of cell division, but it may not be obligatory. A close interpretation of measurements of mass is hazardous in any case, because of the variability of pools, storage synthesis, uptake rates of nutritional substrates, and the other traditional ambiguities of the concept of "growth."

We have little information on the specific kinds of synthesis during division. A deservedly famous experiment of Zeuthen (153) followed the rate of increase in respiratory rate of single *Tetrahymena* in the course of several growth division cycles. He noted a linear increase up to the time of division, an arrest of the increase at the time of division, and a resumption of the original rate of increase by each daughter cell following the interruption. The measurements were made under conditions of optimal growth, and it may be justified to interpret them as measurements of the synthesis of respiratory machinery. If so, this synthesis stops temporarily about the time of division. In studies on *Amoeba* (84), *Tetrahymena* (156), and *Stentor* (28)—all protozoa—it has been found that the rate of incorporation of P^{32} into insoluble compounds decreases markedly during the division period. It is possible (28) that a decline in primary active transport of the precursor into the cell during division may be a major factor; and it is not prudent to conclude that we are observing a decline in the immediate biosynthetic mechanisms. Either interpretation would have the same consequence: a depressed rate of formation of phosphorylated molecules during division.

A recent autoradiographic study of HeLa and leukemic cells exposed to short bursts of tritiated thymidine led to the conclusion that "RNA synthesis stops during mitosis" (31). Similar results have been obtained with cultures of cells of the Chinese hamster (145).

Information on the activity of single enzymes during division is scarce. Relevant studies of D-glyceraldehyde-3-phosphate dehydrogenase and aldolase in lily cells (95) and of peptidase and protease in synchronized yeast cells (141) concur in the finding of a decrease in activity during division.

If the trend of the limited facts is being interpreted correctly, we might conclude that the state of the dividing cell is incompatible with certain kinds of biosynthesis. If this is so, it might be explained in several ways. One line of thought stresses the contrast between the aerobic character of the metabolism of most cells in interphase and the indifference of the dividing cell to oxygen. Another hypothesis (84; critical discussion in 130a) emphasizes the structural changes inherent in mitosis, especially the transformation of the nucleus. The mitotic transformations of the nucleus—the condensation of the chromosomes, the breakdown of the nucleolus, and the breakdown of the nuclear membrane—may, in effect, put the nucleus out of business biosynthetically. If major biosyntheses are under continuous nuclear control, the entrance of the cell into mitosis might have the same consequences as extirpation of the nucleus. The experimental evidence (28, 84) is that enucleation and division do have the same consequences for one crude measure of synthesis—the incorporation of P^{32} into acid-insoluble material. But the structural changes accompanying division are so drastic that cytoplasmic rearrangements may affect the structural basis of biosyntheses. For example, the endoplasmic reticulum, which is supposed to be important in relation to the ribosomal function and, therefore, to protein synthesis, may fragment (106) or at least change its disposition (108) during division.

Thiols.—Thiol groups have demanded attention from students of the

biochemistry of cell division since the 1930's (35, 111, 147). One of the first indications of a major biochemical fluctuation associated with division was Rapkine's (111) finding that "glutathione" (actually TCA-soluble —SH) decreased sharply during the early phases of mitosis in the sea urchin egg and was restored to its original level during the later phases. The "cycle" was interpreted as reflecting the changes in the —SH of structural proteins (reversible denaturation) that accompany the formation of the mitotic apparatus. The glutathione cycle in the sea urchin egg has been reinvestigated by a number of workers (13, 47, 97, 120), and it now seems doubtful that there is a major variation in glutathione concentration itself. In the most thorough of the studies, Sakai & Dan (24, 120) found that a TCA-soluble protein or polypeptide did undergo the cyclic changes attributed by Rapkine to glutathione. Thus, various interpretations based on fluctuations of a "soluble" —SH compound are not ruled out, but only the identification of this compound as glutathione. In lily microspores, glutathione itself does in fact increase before division and decrease during division in a manner that is compatible with Rapkine's findings (129, 130).

The cycle has been interpreted (74, 75) in terms of the hypothesis that intermolecular —S—S— bonding was involved in the assembly of the mitotic apparatus. The mechanism proposed involved, first, a reduction of intramolecular protein S—S with parallel oxidation of glutathione and, then, an oxidation to form intermolecular protein S—S, accompanied by the reappearance of reduced glutathione. However, as has been mentioned above, the weight of the evidence does not favor the original picture of the mitotic apparatus simply as an S—S-bonded molecular polymer. A looser point of view is this: if, as still seems likely, macromolecular interactions involving protein-SH are important in the bonding of the mitotic apparatus, the soluble-SH can certainly participate in the regulation of this bonding (81). Inhibition of division by oxygen at pressures higher than atmospheric has been interpreted in terms of the oxidation of thiol groups (116).

The hypothesis that the connection between glutathione and mitosis might be found in the activation of glycolysis, assuming that glycolysis is closely linked to mitosis (148), has been examined critically by Stern (130). In lily microspores, glycolytic capacity and glutathione concentration were measured during the premitotic and the mitotic period. Far from a direct correlation, an almost exactly inverse correlation between the two measurements was observed. Stern concludes that glutathione is important to mitosis but not in a simple way. In his opinion, it is a "store of radicals and reducing power" meeting the "demands of a number of metabolic channels."

On the physiological side, evidence of a profound involvement of thiols (and perhaps sulfur compounds that are not simply —SH carriers) in cell division continues to accumulate. Nutritional studies on *Chlorella* (37) show that a limitation of the sulfur supply (like sulfates) is first expressed as a specific inhibition of cell division. Under conditions of sulfate deficiency, the cells could grow to some extent and could synthesize DNA but did not go through division. If they were then given sulfate alone (under non-photo-

synthesizing conditions), they divided several times, producing small cells. During the period of nuclear divisions in synchronized populations, an interesting TCA-soluble substance, identified as a sulfur-containing nucleotide-peptide complex, appeared and then declined (38). The system promises the complete identification of one of the molecular links between sulfur metabolism and mitosis. A comparable demonstration of a specific role of sulfur-containing compounds in cell division has been given recently by James and collaborators (52, 100). The flagellates *Astasia* and *Euglena* can be grown successfully on a synthetic medium in which sulfur is supplied as sulfate; the cells do grow and divide. But the time spent in division was remarkably reduced when the medium was supplemented with organic sources of sulfur, notably cysteine and methionine, as though these were crucial and limiting for some process specifically concerned with division.

Thus, one of the earliest insights into the biochemistry of division—the proposal that sulfur-biochemistry plays a special part—continues to be valid and productive, however obscure the actual events may be. It has been useful to distinguish between the “metabolic” and “structural” role of thiol groups in particular experimental situations, but it is doubtful whether either of these classes of mechanisms will be excluded in the end and it is more likely that the distinction will disappear.

CHROMOSOME MOVEMENT

Inference from a great body of microscopic experience has led to no generally acceptable theory of the nature of chromosome movement [Schrader (127)]. The most obvious interpretation of the more typical forms of mitosis is that chromosomes are “pulled” to the poles by fibers in the spindle, but this traction mechanism encounters difficulties in numerous “exceptional” cases. In one form or another, it is still favored by many cytologists. We now have an abundance of accurate measurements of the paths and rates of chromosome movement in anaphase (e.g., 7, 8, 14, 29, 45, 51, 55, 113). While these do not prove the validity of any theory of movement, they do exclude some. For example, it seems doubtful that physical attraction and repulsion in their simplest sense are involved, since the rates of movement are not in accordance with an inverse-square relationship. Novel recent theories include a “jet-propulsion” model (92) and a molecular “pumping” mechanism (99).

All observers now agree that the over-all separation of the chromosomes at anaphase involves two kinds of movements, the magnitude and time-relations of which vary from one kind of cell to the other; one is a movement of the chromosomes to the pole, visually a contraction of the chromosomal fibers. The other is an elongation of the spindle, an increase in pole-to-pole distance.

Biochemical evidence relating to the mechanism of mitotic movements is fragmentary and is influenced to a great extent by the classical hypothesis that the mitotic apparatus (or the dividing cell as a whole) is an analogue of a muscle (66). The most spectacular experiments are those of Hoffmann-

Berling (40, 41), who makes "models" of dividing cells by extracting them with cold glycerol, just as working models of muscle fibers are made by glycerol extraction. In Hoffman-Berling's experiments, at least one component of anaphase movement, the elongation of the spindle, was mimicked when ATP was added to "model" cells in anaphase. The whole cell elongated at the same time. Chromosome-to-pole movements have not yet been observed in such models. Other information relevant to the muscle-mitosis analogy has already been mentioned: the resemblance between the amino acid composition of the mitotic apparatus and that of muscle actin and the association of an ATPase activity with the mitotic apparatus. On the other hand, the evidence does not favor a literal interpretation of the analogy, which says that the various kinds of cellular movement utilize the same contractile molecules, but in different structural arrangements. Antibodies to myosin from chicken muscle do not combine with the mitotic apparatus of chick fibroblasts when tested by the fluorescent-antibody technique (43). Antibodies to the isolated mitotic apparatus of sea urchin eggs do not react with proteins extracted from muscles of adult sea urchins (150). These findings do not exclude close analogies between the two.

Other cytochemical studies deal with the orientation and composition of the mitotic apparatus during the time of chromosome movement. Refinements of polarization microscopy (49) have permitted the certain demonstration that microscopic spindle fibers are "real" structures in the living cell. It is not implied that they are sharply bounded ropes but that there is a distinct alignment of macromolecules corresponding to the chromosome-to-pole and pole-to-pole connections. Important observations are available concerning changes in molecular orientation during anaphase movement (48, 135). Among other changes, it is noted that the region between the separating groups of chromosomes (the interzonal region) is less oriented than the regions between chromosomes and poles. Quite a few observers (e.g., 15, 27, 118), but not all (e.g., 89), find that the interzonal region acquires a higher RNA content than the rest of the mitotic apparatus at the end of anaphase, and this seems to run parallel with an increase in mass as revealed by the interference microscope (69, 118).

Analogies to muscle are useful in the guidance of research on the biochemical background of chromosome movement but do not yet help us explain how chromosomes move, because we cannot really explain how muscles move. If we are dealing with contractile fibrils, certain differences between the "fibers" of the mitotic apparatus, such as the fibrils and filaments of muscle, must be recognized. The former are unstable, are not sharply bounded, and do not shorten or lengthen at constant volume. In fact, we cannot assign them a volume; superficially they seem to be consumed in the process of contraction. The shortening of chromosomal fibers has been visualized in terms of molecular rearrangements involving the loss of some molecules to the "background" and a consolidation of the rest as a shorter element (76). In a recent discussion (48) it has been treated as a

shift in the equilibrium between the oriented and disoriented phase of a molecular population, the oriented phase corresponding to the shortening fiber. Such an image has much to recommend it, but it does not explain how a large body is moved through a resistant medium over a considerable distance. Many workers have stressed the possibility that the chromosome itself is an active participant in its movement; for example, by the release of "structure agents" which bring about a shortening of the adjacent regions of the chromosomal fibers (135). An active role of the chromosomes remains an important possibility.

CYTOKINESIS

It is difficult to formulate the problems of cytokinesis—the division of the cell body following the mitotic separation of the nuclei—in biochemical terms, and little or no information is available. Much descriptive information has become available, especially through electron microscopy. In bacteria the appearance of a transverse membrane has been described (e.g., 21, 151). In plant cells, the initial stages of formation of the new cell wall seem to be related to the movements of endoplasmic reticulum; this collects at the poles early in mitosis, moves through the spindle toward the equator at the time when the chromosomes are moving to the poles, and merges at the equator. The formation of a functional barrier between the daughter nuclei may be related to the fusion of the membranous elements of the endoplasmic reticulum at the equator (108). The furrowing of animal cells has been discussed in terms of various hypotheses involving an active role of the mitotic apparatus, an active expansion of the membrane into the furrow, or the formation of a "contractile ring" at the equator. These hypotheses have been treated in a rather complete review (139). The advocates of the contractile ring (72) view it as a gel that has some of the properties of an actomyosin system, including a participation of ATP in the contraction (61). A great deal of very interesting information on cytokinesis has resulted from physical and experimental studies on living cells, but conventional biochemical attacks are rendered difficult by the fact that only a small part of the cell material is directly involved, whereas the whole cell may be indirectly and intricately involved. We cannot pinpoint an "organ" of cytoplasmic division.

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AUTHOR INDEX

- A
- Abbott, J., 623, 626, 630, 683
- Abdel-Samie, Y. M., 624
- Abd-el-Wahab, A., 174
- Abdullah, M., 355
- Abel-Tawab, G. A., 615
- Abercrombie, M. J., 47
- Abraham, M., 626
- Abraham, S., 206
- Abrams, A., 217
- Abrams, R., 165, 170, 175
- Abramsky, T., 550, 551, 552, 554
- Aht, A. F., 385
- Acevedo, H. F., 505, 507
- Acheson, R. M., 258, 386
- Achor, R. W. P., 395
- Ackermann, D., 580, 583
- Ackmann, R. G., 72
- Acs, G., 299, 300, 301, 307, 311, 312, 318
- Ada, G. L., 349, 364, 375
- Adamiec, A., 156
- Adams, C. W. M., 331
- Adams, D. H., 34
- Adams, E., 256
- Adams, R. A., 627, 630
- Adamson, T. E., 516, 517
- Adelberg, E. A., 660
- Adler, J., 141, 177, 178, 179
- Adlercreutz, H., Jr., 507
- Aebi, A., 86
- Aebi, H., 455, 456
- Aftonomos, L., 619
- Ager, J. A. M., 560
- Agol, V. I., 223, 615, 622, 625, 629
- Agranoff, B. W., 198, 393, 529, 530, 608, 616
- Agre, C. L., 79, 87
- Agrell, I., 678
- Agroskin, L. S., 141
- Aguirre, A., 338
- Ahlquist, L., 74, 79
- Ahmed, F. R., 126
- Ahrens, E. H., 72, 75, 76, 77, 78
- Ajl, S. J., 615, 616
- Akabori, S., 74, 364, 372
- Akabori, Y., 79
- Akasi, S., 75, 76
- Akedo, H., 403
- Akerman, L., 27
- Akesson, A., 276
- Akiya, S., 83
- Alaupovic, P., 460
- Albers, M., 580, 581, 582, 594
- Albers, R. W., 243
- Albert, A., 409
- Alberts, A. W., 193
- Alberts, B. M., 119, 170, 304, 650
- Alberty, R. A., 295
- Albon, N., 353, 358
- Albrecht, G., 96
- Albrecht, M., 73
- Aldag, H. V., 411, 414, 420
- Aldrich, B. B., 477
- Aldrich, P. E., 533
- Aldrich, R. A., 551, 555
- Aldridge, W. J., 649
- Aldridge, W. N., 29, 228
- Alexander, B. H., 46
- Alexander, C. G., 222
- Alexander, H. E., 629, 658
- Alexander, J. A., 511, 516
- Alexander, M., 169
- Alexander, N. M., 562
- Alexander, P., 648, 662
- Alfert, M., 674
- Alfin-Slater, R. B., 462
- Alford, J. A., 73
- Alfred, L. J., 610
- Alfson, A., 374
- Alimova, E. K., 87
- Alioto, M. R., 400
- Allen, B., 258, 556
- Allen, B. K., 177, 440
- Allen, C. H., 329
- Allen, D. A., 560
- Allen, E. H., 297, 299, 300, 301, 303, 304, 307, 312, 315, 318
- Allen, J. R., 21, 461
- Allen, J. T., 662
- Allen, M. B., 465
- Allen, P. J., 354
- Allen, R. S., 453
- Allfrey, V. G., 297, 308
- Allgower, M., 623
- Allison, A. C., 275, 276, 558, 560
- Allison, J. B., 328
- Alonzo, N. F., 84
- Alpert, N. R., 481
- Altman, K. L., 254
- Altman, R. L., 314
- Altschul, A. M., 341
- Altschul, R., 540
- Alvarado, F., 216, 226, 230, 231
- Alver, E., 115
- Amann, R., 597, 598
- Amano, M., 173
- Amano, S., 671, 672
- Ambady, G. K., 100
- Ambe, K. S., 22
- Amberson, W. R., 486
- Ames, B. N., 583, 595, 598, 656, 663
- Ames, S. R., 47, 48, 447
- Amos, H., 305
- Amouch, P., 402
- Amundsen, L. H., 590
- Anagnostopoulos, C., 349, 359
- Anderegg, J. W., 183
- Anderer, A., 560, 561
- Anderer, F. A., 123, 295
- Anders, D. E., 79
- Andersag, H., 431
- Anderson, A. A., 21
- Anderson, C. D., 51, 134
- Anderson, D. G., 541
- Anderson, D. L., 335
- Anderson, E. A., 295
- Anderson, G. W., 143
- Anderson, L., 50, 394
- Anderson, N. G., 184, 599, 643, 673, 677
- Anet, E. F. L. J., 51, 55
- Anfinsen, C. B., 278, 279, 280, 285, 287, 295, 296
- Angell, W., 15
- Angervall, G., 191
- Angyal, S. J., 45, 46, 47, 394
- Anliker, R., 503
- Andreas, H., 75
- Andreasen, A. A., 355
- Andreeva, N. S., 99, 100
- Andrejew, A., 33
- Andrews, J. S., 552
- Andrews, R. V., 615, 629
- Andrews, W. H. H., 221
- Andrus, S. B., 403
- Anthony, W. C., 261
- Antin, J., 457
- Antonini, E., 31, 272, 274, 275, 562, 563
- Antonia, A., 339
- Aoe, N., 473, 479
- Appar, J., 307
- Aposhian, H. V., 241, 316
- Appel, P., 477
- Apple, R. E., 455
- Appleby, C. A., 17, 19, 228, 549, 555
- Applegate, R. L., 183
- Appleman, D., 33
- Aquist, S. E. G., 278
- Arai, G., 126, 490, 491
- Arata, Y., 74
- Archer, B. L., 543
- Arcus, A. C., 73
- Ardizzone, G., 580, 598

- Arena, J., 662
 Arens, A., 358
 Arens, J. F., 450
 Arfin, S. M., 71, 77
 Arrigoni, D., 533, 543
 Arison, B. H., 542
 Armstrong, F. B., 333
 Armstrong, J. McD., 17
 Arnaud, M., 359
 Arnon, D. I., 465
 Arnstein, H. R. V., 432, 439
 Aronow, L., 618, 628
 Aronson, M., 351
 Arrhenius, S. A., 2
 Arrigoni, O., 385
 Arroyave, G., 338, 339, 341
 Arsenaute, G. P., 553
 Arsenijevic, V., 79
 Arthur, W. M., 619
 Arwidsson, B., 34
 Asai, M., 413, 414, 415, 418, 419, 420
 Asakura, S., 475, 476
 Aschaffenburg, R., 102
 Aschner, M., 352
 Asensio, C., 216
 Ashburn, L. L., 597
 Ashby, D. R., 456
 Ashenbrucker, H., 557
 Ashikawa, J. K., 308
 Ashmore, J., 218, 223, 232, 233
 Ashwell, G., 388
 Ashwood-Smith, M. J., 405
 Ashworth, C. T., 189, 190, 191
 Askenazi, I., 232
 Aspinall, G. O., 45, 50
 Assarsson, A., 49
 Asselineau, C., 72, 74, 75, 79, 87
 Asselineau, J., 71, 72, 74, 75, 77, 79, 86, 87
 Astbury, W. T., 100
 Astrachan, L., 315, 317
 Atherden, L. M., 503
 Atherden, S. M., 513
 Atkinson, M. R., 231
 Attardi, G., 624
 Atwater, J., 654
 Atwood, K. C., 180, 650
 Audley, B. G., 411
 Auerbach, V. H., 515, 625
 Augustat, S., 45
 Auhagen, E., 421
 Aures, D., 260
 Auricchio, S., 329, 394
 Austrian, R., 363
 Aveneri-Shapiro, S., 352
 Avi-don, Y., 23
 Avigad, G., 217, 352, 353, 368
 Avigan, J., 536
 Avivi, L., 615
 Avizonis, P. V., 543
 Awapara, J., 243
 Axelrad, A. A., 630,
 Axelrod, A. E., 383, 403
 Axelrod, B., 85
 Axelrod, J., 254, 258, 261, 359, 360
 Axelrod, L. R., 511, 512
 Ayers, J., 287
 Ayers, P. J., 501, 504
 Ayers, W. A., 349, 553
 Azarnoff, D. L., 539
 Azzone, G. F., 486
 B
 Baba, T., 331
 Babcock, M. J., 402
 Bach, S. J., 247, 248
 Bachman, B., 550
 Bachrach, H., 228
 Bachrach, U., 580, 582, 586, 587, 588, 589, 598, 599
 Bachtold, J. B., 614
 Bacila, M., 229, 230
 Bacon, J. S. D., 353, 354
 Baddiley, J., 136, 137, 142, 302, 363, 586
 Bader, A. M., 87
 Baeder, D. H., 204
 Baer, E., 81
 Baer, H. H., 62
 Baev, A. A., 141
 Bagatell, F. K., 175
 Bagby, M. O., 78
 Baggett, B., 501, 507, 508, 512, 515
 Baggett, N., 60
 Baglioni, C., 261
 Bailey, J. M., 371, 609, 611
 Bailey, K., 484, 485
 Bailey, R. E., 506
 Bailey, R. W., 349, 350, 356
 Baird, G. D., 488, 489
 Bajaj, A., 674, 682
 Bakay, B., 663
 Baker, B. R., 51, 64, 133, 134
 Baker, D., 326
 Baker, H., 440
 Baker, R. W. R., 73, 75
 Bakerman, H. A., 253
 Bal, H., 386, 387
 Balakrishnan, S., 261
 Balasundaram, S., 337
 Balatre, P., 394
 Balde, P., 230
 Balderas, L., 501, 507
 Baldwin, M., 207
 Baldwin, D. M., 5
 Bale, W. F., 556
 Balfour, W. E., 506
 Ball, D. J., 51
 Ball, E. G., 13, 190, 191, 204, 205, 206, 222, 223
 Ballantine, J., 23, 464, 465
 Ballard, F. B., 192
 Ballester, C. D., 336
 Ballio, A., 397
 Ballou, C. E., 55, 62, 227, 391, 392
 Bally, P. R., 206
 Balog, J., 274, 560, 561, 562, 564
 Baltazzi, E., 553
 Baltscheffsky, H., 34
 Baltus, E., 296
 Bamford, C. H., 97
 Banerjee, G., 223
 Banerjee, S., 386, 387
 Bangham, A. D., 276
 Banihidi, Z. G., 433
 Banks, J., 34, 517
 Bannerman, M. A., 72
 Bannerman, R. M., 557
 Bao, D. R., 253
 Baptist, N. G., 341
 Baranova, V. Z., 141
 Baranowska, J., 157, 662
 Baranowski, T., 364
 Barany, K., 476, 482
 Barany, M., 476, 482
 Barban, S., 609, 611, 612, 615, 628
 Barbee, K. D., 462
 Barbier, M., 76
 Barchielli, R., 437
 Barclay, R. K., 117, 119
 Barer, R., 122, 678
 Barleau, R. E., 95
 Barkemeyer, H., 420, 421, 423
 Barker, H. A., 201, 438, 439, 440
 Barker, I. R. L., 50
 Barker, N. W., 395
 Barker, R., 49, 62
 Barker, S. A., 45, 46, 49, 50, 58, 62, 65, 348, 349, 350, 351, 365, 369
 Barkhan, P., 387
 Barnard, D., 543
 Barnard, E. A., 278, 279
 Barner, H. O., 617
 Barnes, G. R., Jr., 336
 Barnes, R. H., 465
 Barnes, W. H., 126
 Barnes, L. A., 326
 Barnett, E. V., 618
 Barnett, L., 294, 583, 653
 Barnett, L. B., 282
 Barnett, R. C., 679
 Barnholdt, B., 447, 448
 Baron, C., 34
 Baron, S., 618
 Barone, J. A., 433
 Baronetsky, E., 45
 Bar-Or, R., 586, 588
 Barratt, R. W., 427
 Barrett, J., 15, 19
 Barrionuevo, M., 73
 Barnett, J., 563
 Barnett, R. J., 190, 191, 206, 475
 Barron, E. J., 198

- Barron, G. P., 327
 Barry, B., 556
 Barsky, I. J., 141
 Bartley, W., 24, 25, 26, 34, 83
 Bartsh, R. G., 568
 Bascoulergue, P. R., 341
 Bass, I. A., 658
 Bassett, S. H., 329
 Bassham, J. A., 24
 Bassir, O., 340
 Bastian, R., 284
 Basu, D. K., 36
 Bates, H. M., 300, 307, 308
 Battaile, J., 527, 528
 Battenberg, E., 49
 Bauer, C. D., 337
 Bauer, H., 584
 Baumann, C. A., 395, 535, 540
 Baumann, C. G., 583
 Baumlér, A., 28
 Baykut, F., 71
 Beachtel, W. R., 461
 Beadle, G. W., 293, 294, 641
 Beal, J. L., 544
 Bealing, F. J., 354
 Beamish, R. E., 396
 Bean, R. C., 359
 Bear, R. S., 98, 99, 101, 118
 Beaton, G. H., 387
 Beaton, J. M., 553
 Beatty, I. M., 251, 489
 Beaufay, H., 34
 Beaven, G. H., 274, 277, 284, 560, 561, 564
 Beck, J. C., 506
 Beck, W. S., 201
 Becker, H. J., 433
 Becker, M. M., 372
 Bedell, B. H., 34
 Beek, G., 649
 Beers, R. F., Jr., 156, 166, 167
 Beerstecher, E., Jr., 425
 Beerthius, R. K., 72
 Beetlestone, J., 567
 Beevers, C. A., 115, 125
 Beglioni, C., 653
 Begy, R. W., 29
 Behar, M., 328, 338, 339, 340
 Behavadi, B., 355
 Behrman, E. J., 259
 Beinert, H., 13
 Beiser, S. M., 644, 662
 Bekemeier, H., 455, 456
 Bekker, M. L., 141
 Bélanger, L. F., 457
 Beljanski, M., 317, 318
 Belkin, M., 619, 627, 629
 Bell, D., 179
 Bell, T. T., 427
 Bellamy, D., 249
 Bellamy, D., 249
 Bello, J., 280
 Beloff-Chain, A., 233
 Belozersky, A. N., 141, 158, 316
 Beltz, R. E., 183
 BeMiller, J. N., 52, 54, 55
 Bénard, H., 549
 Bendall, D. S., 34
 Bendall, J. R., 488, 489, 490
 Bendell, J. F., 513
 Bender, A. E., 334, 337
 Bender, H., 358, 364
 Bender, M. L., 57
 Bendich, A., 136, 137, 140, 158, 180, 182, 643, 644, 658, 662
 Bendit, E. G., 101
 Benditt, E. P., 260, 624
 Benesch, R., 276, 558, 559
 Benesch, R. E., 276, 558, 559
 Benhamou, E., 402
 Benhamou, N., 275, 563
 Benirschke, K., 500, 504
 Benitez, A., 134
 Benkers, R., 662
 Bennett, M. J., 251
 Ben-Porat, T., 617
 Bensch, K. G., 606, 620
 Benson, A., 550, 551, 552, 553, 554, 555
 Benson, A. A., 73, 82, 83
 Benson, E. E., 270
 Benson, E. S., 487
 Bensusan, H. B., 486
 Bentley, R., 64, 227, 543
 Benton, F. L., 79
 Benzer, S., 293, 295, 562, 641, 661
 Benzon, M. W., 671
 Ben-Zvi, R., 226
 Berdjis, C. C., 404, 453
 Berends, W., 157, 662
 Berg, A., 49
 Berg, C. P., 250, 330
 Berg, M. H., 551
 Berg, P., 168, 169, 293, 299, 300, 301, 302, 303, 304, 305, 307, 310, 315
 Berge, K. G., 395
 Bergel, F., 36, 431
 Bergen, S. S., Jr., 537
 Bergenstal, D. M., 205
 Berger, L. R., 364, 374
 Bergeret, B., 587
 Bergeron, J. A., 663
 Bergmann, E. D., 536
 Bergmann, F., 36
 Bergmann, F. H., 169, 299, 300, 301, 307
 Bergmann, M., 435
 Bergot, J. L., 341
 Bergquist, A., 657
 Bergstrom, S., 189, 207
 Berlin, N. I., 550
 Berliner, D. L., 503, 505, 510, 511, 512, 618, 625
 Berliner, M. L., 511
 Berman, L., 626, 627, 630, 632
 Bern, H. A., 506
 Bernal, J. D., 95, 102
 Berne, R. M., 21, 22, 490, 491
 Bernfeld, H. C., 595, 600
 Bernfeld, P., 595, 600
 Bernhard, W., 671, 675
 Bernhart, F. W., 326, 404
 Bernhauer, K., 436, 437
 Bernheimer, H. P., 363
 Bernstein, G. S., 228
 Bernstock, L., 86
 Bernst, E., 358
 Berry, J. F., 73, 81
 Berry, M. E., 327
 Berry, R. J., 181
 Berthet, J., 362
 Berti, E., 34
 Bessis, M., 671
 Bessman, M. J., 141, 177, 178, 297, 316
 Best, C. H., 205
 Best, M. M., 396, 540
 Betel, I., 27
 Bethell, J. J., 409
 Bettelstone, J., 14
 Betz, R. F., 177
 Beukers, R., 157
 Bevan, T. H., 81
 Beveridge, J. M. R., 462
 Beychok, S., 275, 558
 Beyer, R. E., 23, 26, 405
 Bharucha, K. E., 78
 Bhate, D. S., 227
 Bhattacharyya, S., 449
 Bhattacharyya, S. C., 79
 Bianco, R., 612
 Bibring, T., 671
 Bichowsky-Slomnitzki, L., 592, 595, 598, 600
 Bickelhaupt, F., 553
 Bickford, W. G., 72
 Bicking, J. B., 434
 Bieber, R. E., 228
 Bieri, J. G., 21, 22, 448, 450, 459, 460, 463
 Bieseke, J. J., 133, 630, 670, 682
 Bieth, R., 73
 Bigelow, C. C., 286, 287
 Biggs, J., 434
 Bigler, F., 86
 Bigwood, E. J., 339
 Bihler, I., 219, 220
 Bijvoet, M. K., 95, 104, 125, 126
 Billen, D., 184, 624
 Billingham, R. E., 631
 Billman, J. H., 580, 598
 Bimes, C., 619, 624
 Bines, B. J., 51, 372
 Bing, R. J., 192

- Birbeck, M. S. C., 101
 Birch, A. J., 415, 543, 544
 Bird, E. W., 458
 Bird, H. H., 632
 Birnbaum, S. M., 125, 330
 Biro, N. A., 476
 Birt, L. M., 24, 25, 26, 34
 Biserte, G., 566
 Bishop, C. T., 45
 Bishop, C. W., 332
 Bishop, J., 312
 Biswas, D. K., 386
 Bitkova, A. N., 141
 Bizzocco, D., 388
 Bjorklund, B., 619
 Bjornstorp, P., 207
 Black, E. C., 473
 Black, J. G., 327
 Black, R. E., 228
 Black R. L., 510
 Black, S., 256
 Black, W. A. P., 65
 Bladon, P., 584
 Blair, M. G., 52
 Blair, R. M., 241
 Blaizot, J., 456
 Blakely, B. R., 616
 Blakemore, W. S., 500,
 501, 502, 506
 Blanchard, P. H., 353, 358
 Blankenhorn, B. E., 486
 Blaschko, H., 586, 587, 588
 Blaylock, B. A., 206
 Blecher, M., 515
 Blewett, M., 537
 Bloch, D. P., 663
 Bloch, E., 500, 502, 504
 Bloch, K., 194, 195, 196, 300,
 318, 390, 528, 531, 533, 535,
 537
 Block, R. J., 334, 337
 Bloemendal, H., 169
 Blohm, T. R., 536
 Blomstrand, R., 190
 Bloom, B., 515
 Bloomfield, D. K., 195, 196,
 390
 Blow, D. M., 104, 105
 Bluhm, H. M., 491
 Bluhm, M. M., 102, 104, 105
 Blum, J. J., 260, 481
 Blumberg, A., 455, 456
 Blumenfeld, L. A., 141
 Blumenfeld, O. O., 285, 286
 Blumsom, N. L., 142, 363
 Bo, W. J., 451
 Boardman, N. K., 567
 Boccacci, M., 454
 Bock, F. G., 490
 Bock, R. M., 12, 357
 Bodansky, O., 34, 228
 Bodo, G., 102, 104, 105, 107,
 270, 564, 566
 Boedtker, H., 158, 304, 310,
 648, 649
 Boemendal, H., 311
 Boeri, E., 17
 Boettiger, E. G., 473
 Boeuf, B., 205
 Boeye, A., 661
 Bogdonoff, M., 204, 205
 Boggiano, E. M., 255
 Bogorad, L., 550, 551, 552,
 553, 554, 555, 567
 Bogoyavleskaya, N. V., 165
 Bohn, H., 580, 581, 583
 Boigné, N., 400
 Boisselot-Lefebvres, J., 387
 Bokhoven, C., 104
 Bolaffi, J. L., 617
 Bolayir, S. T.
 Boldrini, R., 404
 Bolen, R. J., 457
 Bolgova, G. D., 87
 Bolinder, A., 176
 Bolinger, R. E., 539
 Bollum, F. J., 179, 180, 183,
 650
 Bolognari, A., 681
 Bolton, E. T., 302
 Boman, H. G., 299, 300,
 301, 307, 311, 312
 Bonavita, V., 242, 400, 402
 Bond, H. E., 677
 Bond, P. A., 244
 Bond, V. P., 172, 174, 617,
 621, 680
 Bondi, A., 447
 Bondy, P. K., 506
 Bondy, S. C., 473
 Bongiovanni, A. M., 504,
 506
 Bonhoeffer, F., 158
 Bonner, J., 309, 431, 543
 Bonner, T. G., 61
 Bonner, W. A., 65, 683
 Bonner, W. D., 568
 Bonnett, R., 435, 553
 Bonsignore, A., 232
 Bonting, S. L., 625
 Bootj, H. L., 553, 556
 Booker, L. K., 329
 Booker, R. K., 329
 Booth, A. N., 256, 257
 Booth, V. H., 388
 Borchers, R., 336
 Borders, D., 580, 598
 Bordet, C., 75, 78
 Borecky, J., 72
 Borenfreund, E., 662
 Borer, R., 393
 Boretti, G., 437
 Borgström, B., 189, 190,
 191, 192, 207, 326, 354
 Borman, A., 536
 Born, J., 226
 Borooah, J., 374
 Borrebaek, B., 222
 Borsook, H., 296, 312
 Borst, P., 28, 249
 Bosch, L., 169, 311, 617
 Boschman, T. A. C., 375
 Bose, J. L., 47, 48, 50
 Boss, J., 674, 682, 683
 Bossenmaier, I., 551
 Bostic, W. L., 611
 Bots, J. P. L., 455
 Botticelli, C. R., 507
 Botts, J., 481, 483
 Bouchilloux, S., 259
 Boulter, D., 543
 Bouman, J., 460
 Bourgeois, S., 247
 Bourne, E. J., 46, 49, 61
 Bourne, E. J., 46, 49, 61
 Bourquelet, E., 372
 Bouscayrol, S., 560
 Bouveng, H. O., 45
 Bovard, F. C., 299, 300
 Bovey, F. A., 286, 287,
 349, 350, 371
 Bowen, W. J., 480, 483,
 487
 Bowering, W. D. S., 65
 Bowerman, A. M., 503
 Boxer, G. E., 175, 233
 Boyer, A., 326, 329
 Boyer, P. D., 11, 227, 360,
 549
 Boyland, E., 597
 Brachet, J., 172, 293, 296,
 298, 308, 314, 663, 671,
 677
 Bradbeer, C., 196, 239
 Bradbury, E. M., 99
 Bradley, D. F., 157, 491,
 647, 650
 Bradley, D. K., 449
 Bradley, R. M., 198
 Bradley, S. G., 234, 609,
 628
 Bradlow, H. L., 511
 Brady, R. O., 192, 195, 360
 Braekkan, O. R., 448
 Braganca, B. M., 426
 Bragdon, J. H., 190
 Bragg, P. D., 63
 Bragg, W. L., 102, 105
 Brahms, J., 480, 481, 484
 Braithwaite, G. D., 540, 541,
 542
 Brand, K. G., 619, 632
 Branson, H. R., 97, 100,
 108
 Bras, G., 339
 Braude, R., 394
 Braun, J. V., 584
 Braunitzer, G., 111, 275,
 561
 Braunstein, A. E., 244, 491
 Braverman, G., 172, 302
 Bray, H. G., 256
 Bray, R., 435
 Bray, R. C., 36
 Breck, M., 50
 Breckenbridge, B. M., 362
 Bremer, J., 81, 255
 Bremner, J. M., 580
 Brennan, K., 261
 Brenner, S., 122, 124, 294,

- 316, 583, 653
 Brenner- Holzach, O., 409, 412
 Bresler, A. E., 73, 74, 169, 171
 Bresler, S. E., 158
 Breslow, E., 158
 Breslow, R., 258, 386, 433, 435
 Bresnick, E., 366
 Bressani, L. R., 328, 338
 Breton-Gorius, J., 671
 Breuer, H., 510, 511, 512, 513
 Breuer, H. J., 354
 Bridel, M., 372
 Briggs, F. N., 487, 488, 489
 Briggs, G. M., 463
 Briggs, M. H., 257
 Brill, A. S., 31
 Brimacombe, J. S., 58, 59, 60, 61, 63
 Brin, M., 402
 Brinigar, W. S., 14
 Britton, H. G., 221
 Broadley, J. S., 126
 Broberg, P. L., 411, 412
 Brock, J. F., 328, 337
 Brock, M. L., 215
 Brock, T. D., 215
 Brockerhoff, H., 78, 198
 Broda, E., 615, 624
 Brodie, A. F., 23, 462, 464, 465
 Brodie, B. B., 258
 Brodie, J., 526, 527
 Brodsky, V. J., 141
 Broker, T. N., 658
 Brönd Larsen, J., 450
 Bronk, J. R., 29
 Brookes, P., 138
 Bro-Rasmussen, F., 447
 Brossmer, R., 375
 Broughton, M. E., 73
 Brown, A., 631
 Brown, A. D., 593
 Brown, A. V. W., 307, 646
 Brown, B., 250
 Brown, D. D., 253, 254
 Brown, D. E. S., 482
 Brown, D. H., 226, 231, 362, 363, 364
 Brown, D. J., 36
 Brown, D. M., 82, 133, 136
 Brown, E. G., 410, 412, 419, 550
 Brown, G. B., 133, 134, 137, 138
 Brown, G. L., 140, 303, 304, 307, 646
 Brown, G. M., 391, 397, 409, 423, 424, 425, 428, 428, 429, 430, 431, 433, 434
 Brown, G. W., Jr., 201, 248
 Brown, H. E., 338
 Brown, J., 228
 Brown, J. D., 516
 Brown, J. W., 337
 Brown, L., 97, 98
 Brown, M. L., 403
 Brown, P. K., 451
 Brown, R. H., 203, 204, 205, 206
 Brown, R. K., 278, 281
 Brown, R. R., 261
 Brown, S. J., 243
 Brown-Grant, K., 509
 Brubacher, G., 449, 464
 Bruce, G. T., 349, 350
 Bruce, I. C. L., 57
 Bruce, V., 643
 Bruemmer, J. H., 239
 Brüggemann, J., 449
 Bruggen, J. T., 206
 Brummond, D. O., 361
 Brunius, E., 447
 Bruns, F. H., 227
 Brush, M., 402
 Bruun, T., 76
 Bryan, A. M., 672
 Bryant, J. C., 610, 628
 Bryson, M. J., 503, 505, 512, 618
 Brzezinsky, A., 580, 598
 Bubl, E. C., 47, 48
 Bublitz, C., 199, 201
 Buc, H., 86
 Buccino, R., 388
 Buchanan, A. A., 625
 Buchanan, J. G., 136, 137
 Buchanan, J. M., 165, 255, 297, 316, 412, 440
 Buchanan, M. A., 72
 Bucher, N. L. R., 526, 531, 671
 Bücher, T., 24, 233
 Buchman, E. R., 431
 Buchnea, D., 81
 Buchschacher, P., 553
 Budnick, L. E., 515
 Budowitch, T., 355
 Budowski, P., 447
 Bueding, E., 226, 260
 Buell, G. B., 190
 Buerger, M. J., 126
 Buettner-Janusch, V., 142
 Bullock, E., 15, 553
 Bullough, W. S., 672, 678, 679
 Bu'Lock, J. D., 76
 Bunim, J. J., 510
 Bunina, N. N., 141
 Bunton, C. A., 47
 Bunville, L. G., 647
 Bunyan, J., 22, 458, 459
 Burch, H. B., 339
 Burdon, J., 62, 66
 Burdon, R. H., 168, 170
 Burg, R. W., 401
 Burge, R. E., 99, 100
 Burger, M., 216, 218, 456
 Burgers, A. C. J., 506
 Burgess, E. A., 34
 Burgi, E., 646, 647
 Burk, D., 630
 Burley, J., 512
 Burlington, H., 625, 626
 Burma, D. P., 36
 Bunness, A. T. H., 649
 Burns, J. J., 383, 384, 388
 Burns, S. N., 660
 Burns, V., 671
 Burr, W. W., Jr., 190
 Burris, R. H., 32, 239, 568
 Burstein, S., 511
 Burt, A. M., 611
 Burton, K., 295, 316, 486, 642
 Burton, R. M., 360
 Bush, I. E., 510, 511
 Bussard, A., 314, 315, 316
 Butenandt, A., 76
 Butler, D. N., 137
 Butler, G. C., 51, 513
 Butler, H. C., 507, 512
 Butler, J. A. V., 645, 648, 649, 663
 Butler, R., 540
 Butterfield, W. J. H., 206
 Buzzanca, P., 457
 Buzzell, A., 184, 662
 Byers, S. O., 204
 Byrne, R., 118
 Byrne, W. L., 231, 251

C

- Cabannes, R., 560
 Cabib, E., 142, 360, 363
 Cacioppo, F., 457
 Cadenas, E., 218, 219, 221, 226, 230
 Cahill, G. F., Jr., 192, 204, 205, 206, 222
 Cailleau, R., 172, 605
 Cain, D. F., 486, 487
 Cairncross, I. M., 50
 Cairns, J. F., 567
 Caldwell, M. L., 372
 Calkins, D. G., 437
 Callen, J. E., 72
 Callow, R. K., 76
 Calvanese, N., 403
 Calvin, M., 228, 271, 283
 Cama, H. R., 337
 Camba, R., 580
 Cameron, C., 259
 Camiener, G. W., 428, 429, 430, 431, 433
 Camp, J. L., 623
 Campbell, D. J., 454
 Campbell, J. A., 334, 335
 Campbell, L. L., 249
 Campbell, P. N., 308
 Campbell, P. M., 293
 Campbell, R. C., 183
 Canellakis, E. S., 187, 168, 169, 175, 176, 180, 301,

- 303, 304, 315
 Cannon, J. R., 435
 Cantley, M., 47
 Cantoni, G. L., 158, 167,
 169, 255, 303, 304, 586,
 595, 596
 Capon, B., 45
 Caputo, A., 272, 274, 275,
 562, 563
 Caputto, R., 384, 461, 489,
 490
 Carafoli, E., 486
 Caravaca, J., 247, 248
 Carbone, J. V., 360
 Cardini, C. E., 347, 359,
 360, 362
 Carleton, A., 385
 Carlisle, C. H., 102
 Carlson, F. D., 487
 Carlson, G. L., 434
 Carlson, L., 679
 Carlstrom, D., 126
 Carminatti, H., 223, 362
 Carnahan, J. E., 239
 Carol, E., 338
 Carpenter, A. T., 555
 Carpenter, K. J., 336, 394
 Carpenter, M. P., 384,
 461, 482, 489, 490
 Carrere, C., 359
 Carroll, B. R., 510
 Carroll, W. R., 35, 477
 Carruthers, C., 28
 Carstensen, H., 506
 Carta, R., 580
 Carter, A. C., 500, 503
 Carter, H. A., 615
 Carter, H. E., 74, 82
 Carter, J. R., 280, 282
 Carter, P., 512
 Cartwright, G. E., 557
 Carvajal, E. J., 589, 599
 Carvajal, G., 589, 599
 Case, J. D., 261
 Case, M. E., 657
 Caserio, M. C., 64
 Cason, J., 72, 79, 87
 Caspar, D. L. D., 121, 122,
 123, 124
 Caspersson, T. O., 679
 Caspi, E., 510
 Cassman, M., 517
 Castellfranco, P., 33, 300
 Castellucci, N. T., 79
 Castillo, R., 334
 Castle, J. E., 239
 Castor, C. W., 623
 Castor, L. N., 13
 Cate, D. L., 190
 Cathou, R. E., 255, 440
 Cattaneo, S. M., 182
 Catron, D. V., 452, 453
 Caughey, W. S., 551
 Causseret, J., 456, 457
 Cava, E. E., 395
 Cavalca, L., 97
 Cavalieri, L. F., 156, 158,
 643, 645, 646, 648
 Cavallero, F., 8
 Cavallini, D., 242
 Cavanaugh, M. W., 623,
 683
 Cavelli, I., 245
 Cavina, G., 447
 Cawkwell, J. M., 479, 491
 Cecil, R., 276, 558
 Cekleniak, W. P., 207
 Ceriotti, G., 34
 Černý, M., 62
 Cestari, A., 615
 Cha, C.-Y., 285
 Chadwick, B. T., 260
 Chadwick, J. M., 387
 Chalet, A. H., 388
 Chalet, L., 388
 Chaikoff, I. L., 201, 206,
 207, 234, 502
 Chaix, P., 16, 28
 Chakravarty, K. K., 79
 Chalkley, H. W., 681
 Chambers, R. W., 143,
 165, 482
 Chan, P. C., 26, 425
 Chance, B., 11, 13, 18, 20,
 26, 27, 28, 233, 486, 568
 Chang, C. D., 45
 Chang, R. S., 608, 609,
 615, 616, 628
 Chang, S. C., 425
 Chang, Y., 383
 Chanley, J. D., 51
 Chantrenne, H., 172, 293,
 296, 298, 314, 315, 316
 Chapman, D., 74
 Chapman, D. G., 334
 Chapman, G. B., 684
 Chapman, P. J., 257
 Chappell, J. B., 26
 Charalampous, F. C., 75,
 393
 Chardenet, P., 451
 Chargaff, E., 172, 178, 295,
 304, 310, 315, 642
 Charlwood, P. A., 274, 564
 Chatagner, F., 405
 Chatterjee, B. G., 45
 Chatterjee, G. C., 383
 Chatterjee, I. B., 383
 Chatzinoff, A., 451
 Chaudhuri, D. K., 394
 Chaudhuri, N. K., 617
 Chayen, J., 674
 Chayen, R., 71
 Chaykin, S., 528
 Cheesman, D. F., 484
 Chen, M., 333
 Chen, Y. T., 383
 Cheng, P., 644
 Cheng, P.-Y., 309, 316
 Cheo, P. C., 649
 Cheong, L., 137, 617
 Cherkashina, N. A., 141
 Cherkes, A., 191
 Chernick, S. S., 461
 Chernoff, A. I., 561
 Chernova, Z. A., 141
 Chernukh, A. M., 400
 Chervinka, C. H., 286
 Cheslock, K. E., 402
 Cheung, M. W., 339
 Chèvremon, M., 671, 678
 Chiang, C., 229, 230
 Chiang, J. L., 388
 Chiba, T., 597
 Chichester, C. O., 541
 Chinard, F. P., 221
 Chirigos, M. A., 241
 Chisholm, J. J., 78
 Chisholm, M. J., 75, 76,
 77, 78
 Chmielewska, J., 23
 Chobanian, A. V., 536
 Chopard-dit-Jean, L. H.,
 463
 Chouroulinkov, H., 630
 Chow, B. F., 402, 404,
 438
 Chow, T.-L., 57
 Chowes, I., 192, 205
 Christensen, H. N., 242,
 821
 Christensen, K., 45
 Christensen, N. O., 448,
 455
 Christie, S. M. H., 143,
 147
 Christophe, J., 531
 Chu, E., 671
 Chu, E. H. Y., 627, 630
 Chu, E. J. H., 551
 Chu, T. C., 551
 Chuckler, A. C., 433
 Chung, C. W., 168, 170
 Chung-Ing, L., 141
 Chursina, K. I., 584
 Chvapil, M., 254
 Ciaccio, E. I., 233
 Cieciura, S. J., 627, 628
 Cifonelli, J. A., 363
 Ciotti, M. M., 228
 Civen, M., 246, 515
 Claff, C. L., 677
 Clancy, M. J., 63, 372
 Clapper, W. E., 412
 Clark, A. H., 77
 Clark, A. J., 537
 Clark, B., 81, 190, 197
 Clark, C. T., 258, 260
 Clark, H. E., 327, 329
 Clark, R. L., 433
 Clark, S. L., Jr., 189
 Clark, V. M., 143, 146
 Clark, W. M., 11
 Clarke, D. D., 584, 590
 Clarke, R. T. J., 356
 Clark-Lewis, J. W., 253
 Classen, L. J., 455
 Clegg, M. D., 259, 276,
 560
 Cleland, M., 526, 528,
 532, 538

- Cleland, M. G., 487
 Cleland, W. W., 360
 Clements, L. M., 580
 Clerk, B. J., 281
 Cleveland, F. C., 371
 Cleverley, B., 74
 Clews, C. J. B., 115
 Clezy, P., 15, 19, 563
 Cline, R. E., 138
 Closs, G. L., 553
 Clouet, D. H., 30
 Clowes, R. C., 641
 Čmucharová, B., 254
 Coalson, R. E., 623
 Coates, J. H., 647
 Cobb, J. P., 623
 Cochran, W., 94, 95, 115
 Codrington, J. F., 136
 Coen, L. J., 241, 403
 Cohen, A., 502, 582, 598, 599
 Cohen, C., 101, 473, 478
 Cohen, F., 333
 Cohen, G. N., 214, 216, 293, 302
 Cohen, H. P., 386
 Cohen, J., 100
 Cohen, J. A., 183
 Cohen, P. P., 247, 248
 Cohen, S., 341, 536
 Cohen, S. S., 196, 297, 308, 315, 316, 581, 582, 594, 617, 642
 Cohen-Basire, G., 358
 Cohn, C., 327
 Cohn, G. L., 500, 504, 506
 Cohn, M., 216, 348, 350, 357, 358, 364, 366, 624
 Cohn, W. E., 47
 Coirre, J., 372
 Coker, R., 490
 Colacurci, A., 598
 Colarusso, R., 454
 Colas, A., 513
 Colas, M. C., 394
 Cole, A. R. H., 58
 Cole, D., 558
 Cole, E. W., 334
 Cole, L. J., 649
 Cole, R. D., 300
 Coleman, D. L., 535
 Colle, E., 512
 Collil, W., 16
 Collins, F. D., 74
 Collis, W. R. F., 339
 Colman, R. F., 244
 Colowick, S. P., 364
 Colpa-Boonstra, J. P., 18, 20, 465
 Colpa-Boonstrap, J., 19, 20
 Colter, J. S., 632
 Colvin, J. R., 274, 563
 Comb, D. G., 142
 Comline, R. S., 506
 Comly, L. T., 311
 Commoner, B., 24, 122
 Concepcion, I., 337
 Conchie, J., 374
 Coniglio, J. G., 190
 Conn, E. E., 244
 Connell, G. E., 560
 Connell, J. J., 477, 480
 Connelly, C. M., 486
 Connelly, J., 15, 19
 Conney, A. H., 383, 384
 Conover, T. E., 23
 Conquy, T., 451, 452
 Conrad, H., 14, 567
 Constable, B. J., 387, 388
 Cook, B., 629
 Cook, R., Jr., 625
 Cook, T. M., 22
 Cook, W. H., 138, 139, 141, 142
 Cookson, G. H., 553, 554
 Coon, M. J., 199, 330, 526
 Cooper, C., 26
 Cooper, D. Y., 500, 501, 502, 506
 Cooper, F. P., 45
 Cooper, J. A. D., 254
 Cooper, O., 13, 223
 Cooper, P. D., 611
 Coots, J., 300
 Collins, A., 551
 Copeland, W. H., 355
 Coppola, J., 126
 Coraggio, F., 612
 Corbett, K., 248
 Corbridge, D. E. C., 115
 Corcoran, J. W., 435
 Cordes, E., 140, 304, 307
 Corey, R. B., 95, 96, 100, 101, 114
 Cori, C. F., 218, 364, 371
 Cori, G. T., 348, 351, 356, 362, 364
 Cori, O., 490
 Coriell, L. L., 629, 632
 Cormier, M., 73
 Cornforth, J. W., 528, 529, 530, 534, 535, 540, 541, 544
 Cornforth, R. H., 531, 532, 534
 Corse, J., 72
 Corsi, A., 474, 484, 485, 486
 Corwin, A. H., 552, 553
 Corwin, L. M., 21, 26, 27, 481
 Costamailere, A. L., 336
 Costelli, J., 480
 Costello, R. L., 539
 Cotereau, H., 587
 Cottone, M. A., 73
 Cotzias, G. S., 245
 Courtois, J. E., 47, 349, 357, 358, 359
 Cowan, J. C., 79
 Cowan, P. M., 98, 99
 Cowan, R. L., 327
 Cowgill, R. W., 226, 399, 400
 Cowie, D. B., 302
 Cox, E. G., 125
 Cox, R. A., 158, 303, 304, 310
 Cox, R. I., 502, 503
 Craddock, V. M., 308
 Craig, B. M., 72, 78
 Craig, J. M., 627, 630
 Craig, L. C., 275, 558, 561
 Cramer, F., 57
 Crammer, J. L., 284
 Crampton, C. F., 663
 Crandall, D. I., 257
 Crandall, R. E., 412
 Crane, C. W., 332
 Crane, F. L., 22, 567
 Crane, H. R., 650
 Crane, R. K., 218, 219, 220, 226
 Crasemann, J. M., 662
 Cravioto, J., 338, 339, 340, 341
 Cravioto, O. Y., 336
 Cravioto, R. O., 336
 Crawford, E. J., 227, 362
 Crawford, R. B., 22, 459
 Crawhall, J. C., 251
 Creasey, W. A., 181
 Creighton, A. M., 51
 Cresswell, R. M., 413, 415
 Creveling, C. R., 257
 Crick, F. H. C., 93, 94, 98, 99, 100, 101, 102, 105, 117, 118, 124, 177, 188, 293, 295, 299, 310, 312, 642, 643, 647, 651, 658, 661
 Criddle, R. S., 12
 Criddle, W. J., 65
 Crillo, V. P., 216
 Crocker, T. T., 172
 Crook, E. M., 356
 Croon, J., 65
 Crosbie, G. W., 243
 Cross, M. J., 255, 439, 440
 Crowe, P. F., 72
 Crowell, B. B., 34, 232, 653
 Cruickshank, D. W. J., 126
 Cruickshank, W. M., 456
 Crum, J. D., 49
 Crute, M. B., 126
 Cruz-Coke, E., 458
 Csaky, T. Z., 220
 Csallany, A. S., 21, 22, 459, 542
 Culik, R., 332
 Cullis, A. F., 104, 269, 271, 559, 560
 Cummins, C. S., 86
 Curran, G. L., 195, 198, 539
 Curran, J. F., 244, 502, 517
 Curtis, E. J. C., 63
 Cusworth, D. C., 326

D

- Dabrowska-Piadowska, K., 626
 D'Adamo, A. F., Jr., 233
 Dadd, R. H., 387
 D'Adda, I., 8
 Dagley, S., 257, 308
 Dahlback, O., 190
 Dahlquist, A., 220, 354, 355
 Dahms, G., 426
 Dakrouy, A. M., 332
 Dakshinamurti, K., 391
 Dales, S., 611, 615, 619, 663
 Dalglish, C. E., 296
 Dallam, R. D., 27, 29
 Daly, J. W., 245, 258
 Dam, H., 208, 462, 463, 464
 D'Amato, E., 326, 404
 Dan, K., 681, 675, 676
 Danes, B. S., 615
 Danforth, W. H., 192
 Daniel, M., 615
 Danielson, W., 537
 Danielsson, H., 207, 208
 Danilshafsky, I., 63
 Dannenberg, H., 13
 Danzig, M., 584
 Darby, W. J., 325, 387
 Darke, S. J., 333
 Darnell, J. E., Jr., 613, 622, 629
 Das, N., 674
 Dassler, C. G., 56, 62
 Datta, A. G., 232
 Dauchy, S., 75, 77, 87
 Daune, M., 275, 563
 Davenport, H. E., 568
 Davern, C. I., 315
 Davey, C. L., 490, 491
 David, S., 51
 David, S. F., 619, 624
 Davidson, D., 599
 Davidson, E. A., 226, 231
 Davidson, I. W. F., 205
 Davidson, J. D., 628, 629
 Davidson, J. N., 179, 180, 616, 618, 620, 621, 631, 650
 Davidson, N., 559, 647
 Davie, E. W., 300, 301, 318
 Davies, D. A. L., 45
 Davies, D. R., 102, 106, 108, 109, 115, 117, 118, 119, 269, 270, 271, 559, 560, 650
 Davies, H. A., 46
 Davies, H. G., 683
 Davies, J. R., 625
 Davies, R. E., 486, 487
 Davies, R. K., 248
 Davis, B. D., 250, 256, 440, 463, 465
 Davis, E. V., 609
 Davis, F. T., 649
 Davis, V. E., 243
 Davison, A. N., 12, 260
 Davison, P. F., 158, 641, 643, 644, 646, 648
 Davoll, J., 133, 134
 Dawes, G. S., 597
 Dawkins, M. J. R., 26, 29
 Dawson, A. M., 190
 Dawson, B., 95
 Dawson, R. M. C., 391, 392
 Day, A. J., 192
 Day, M., 624
 Day, P. L., 177, 440, 461
 Dayal, B., 126
 Dayton, J., 205
 Dayton, P. G., 384
 Dayton, S., 205
 Dean, F. D., 507
 Dean, R. F. A., 338, 339, 340
 Deborin, G. A., 141
 Debuch, H., 82, 199
 de Coninck, S., 34
 Decoteau, A. E., 75
 DeCourcy, C., 503, 511
 Dedonder, R. A., 347, 348, 352, 353, 365, 367, 368, 371
 de Duve, C., 34
 Deeds, F., 256, 257
 De Falco, A., 248
 Default, C., 451
 Defendi, V., 631, 632
 de Garihe, M. P., 154
 Degelth, R., 96, 105
 DeGoodman, W. S., 531
 De Groot, N., 249
 de Haas, G. A., 81
 DeHarven, E., 671, 675
 Deinema, M. H., 87
 Deitrich, R. A., 205
 Dekker, C. A., 139, 156, 165, 643
 Dekker, E. E., 254, 526
 deKloet, S. R., 318
 De Kruij, P., 2
 de la Fuente, G., 216, 228
 Delaney, R., 281
 Delbrück, A., 233
 Dell Campillo, A., 199, 526
 Delluva, A. M., 486, 487
 Dellweg, H. W., 436
 de Luca, C., 144
 DeMaeyer, E. M., 338, 339
 Demain, A. L., 373
 De Man, T. J., 448, 449
 DeMarco, C., 242
 DeMars, R., 607, 612, 618, 627
 Demarteaue-Ginsburg, H., 85
 Demerec, M., 656, 660
 Demos, J., 475
 DeMoss, J. A., 300
 de Mowbray, R. R., 251
 Dempsey, B., 556
 Den Hartog, H., 31
 Dennis, D., 34
 Deranche, R., 51
 Derbes, V., 338
 de Robichon-Szulmajster, H., 216, 231, 302
 Derrien, Y., 560, 561
 Derse, P. N., 334
 De Santis, P., 115, 116
 DesArmier, R. M., 614
 Desaulles, P., 506
 Deshpande, M. M., 79
 DeSilva, C. C., 341
 Desnuelle, P., 198
 DeTar, D. F., 434
 De Terra, N., 680
 Deuel, H., 373
 Deul, D., 460
 Deutsch, J. F., 158, 648
 de Villafranca, G. W., 474
 Devlin, T. M., 34
 Devreux, S., 315
 De Vries, A., 192
 de Waard, A., 527, 528
 DeWall, R. A., 490
 Dewar, E. T., 65
 De Zotti, G., 73
 Dialameh, G. H., 542, 543
 Diamanstein, T., 230
 Diamantis, A. A., 239
 Diaper, D. G. M., 584
 Diara, A., 87
 Dick, E. C., 537
 Dicke, W. K., 220
 Dickel, G. B., 79
 Dickerman, H., 24, 397
 Dickerson, R. E., 106, 269, 270, 271, 559, 560
 Dickinson, A. M., 433
 Dickman, S. R., 277, 278
 Dicks, M. W., 449
 Dickson, M., 631
 Dickson, W. M., 507, 512
 Dieczfalusy, E., 507
 Dieckert, J. W., 72
 Dieckmann, M., 169, 299, 300, 301, 303, 304, 305, 307, 310, 315
 Diehl, H. W., 51
 Diehl, J. F., 461
 Diener, T. O., 658
 Dienhardt, F., 615, 629
 Dierks, C., 282
 Dietel, B., 628
 Dieterle, J. M., 447
 Dietrich, L. S., 397
 Dietsche, W., 583
 Dijkstra, G., 72
 Dikshit, P. L., 457
 Dikstein, S., 228
 DiMarco, A., 437
 Di Marco, B., 437
 DiMartinis, J., 510
 DiMayorca, G. A., 658
 Dimler, R. J., 46
 Dingle, J. T., 615
 Dingman, W., 248
 Dinh-Nguyen, N., 74

- Dinning, J. S. 177, 246,
 440, 441, 461, 490
 Dintzis, H. M., 102, 104,
 105, 107, 270, 310
 Dioguardi, N., 333
 Dion, H. W., 437
 Diplock, A. T., 22, 458,
 459, 541
 Diringer, R., 171, 563
 Dirksen, M. L., 643
 DiSabato, G., 26
 Diskina, B. S., 141
 Dittmer, J. C., 391, 392
 Dixon, G. H., 296
 Dixon, G. J., 487
 Djordjevic, B., 618
 Dmuchowski, B. L., 63
 Dobinson, B., 62
 Dobson, H. L., 21, 461
 Doctor, B. P., 140, 307,
 593
 Dodgson, K. S., 63, 374
 Doeg, K. A., 18
 Doell, R. G., 317
 Doerr, I. L., 134
 Doisy, E. A., 207, 501
 Doisy, E. A., Jr., 501
 Dokos, J. M., 605, 625
 Dole, V. P., 180, 205
 Doljanski, L., 625, 626
 Domagk, G., 175, 616
 Dominguez, O. V., 505,
 507
 Donaldson, K. O., 21, 22,
 425, 460
 Doniach, D., 619
 Donis, O., 464
 Donohue, J., 95, 96, 119
 Donovan, J. W., 286
 Donyan, G., 24
 Dordahl, L. A., 206
 Dorfman, A., 363
 Dorfman, R. I., 501, 503,
 504, 507, 508, 509, 511,
 512
 Dornberg, M. L., 125
 Dorrien, U., 679
 Dorsey, M., 626
 Dosa, A., 580
 Doty, P., 118, 119, 158, 170,
 184, 269, 270, 277, 282,
 283, 284, 288, 296, 309,
 316, 304, 310, 644, 645,
 646, 647, 648, 649
 Doudoroff, M., 87, 348,
 365, 367, 368, 369
 Dougall, D. K., 15
 Dougherty, T. F., 510, 511,
 618, 625
 Dounce, A. L., 296, 663
 Dove, W. F., 647
 Dowling, J. E., 448, 450,
 451
 Downing, D. T., 73
 Doy, C. H., 247, 258, 259
 Doyme, T., 95
 Drabkin, D. L., 587
 Drain, G. F., 483
 Draper, H. H., 21, 22,
 459, 462, 542
 Drechsler, E. R., 227
 Dreisbach, L., 73
 Dresel, E. I. B., 550, 553,
 554, 555, 556
 Drew, R. M., 617
 Dreyer, W. J., 294, 653
 Dreyfus, J. C., 312, 317,
 486, 560
 Drimmer, F., 205
 Drolet, B. P., 605, 625,
 630
 Drozdov, N. S., 79
 Drummond, G. I., 199, 473
 Drysdale, G. R., 35
 Drysdale, R. B., 618
 Dryselius, E., 56
 Dubin, D. T., 580, 581,
 582, 583, 584, 587, 588,
 590, 595, 598, 599, 663
 DuBois, R., 339
 Dubos, R. J., 580, 599
 Dudley, H. W., 584
 Duerksen, J. D., 356, 370
 Duggan, D. E., 458
 Dugros, E. C., 36
 Duguid, J. P., 684
 Dulbecco, R., 629, 641, 642
 Duncan, C. H., 396, 540
 Duncan, G. W., 503
 Duncan, H., 11
 Duncan, R. E., 682
 Duncan, W. A. M., 356
 Dunell, B. A., 74
 Dunn, D. B., 303, 304, 305,
 310
 Durell, J., 255, 586
 Durieux, J., 281
 Durr, I. F., 526
 Dus, K., 566
 Duschinsky, R., 133
 Dutler, H., 553
 Dutra de Oliveira, J. E.,
 387
 Dutta, B. N., 526, 538
 Dutton, A. H., 624
 Dutton, G. G. S., 65
 Dutton, G. J., 360
 Dutton, H. J., 74
 Dutton, R. W., 624
 Dvorak, J., 79
 Dvorak, Z., 490
 Dyrenfurth, I., 506
 Dzgurov, S. G., 625
- E
- Eagle, H., 331, 393,
 605, 606, 607, 608,
 609, 610, 611, 612,
 613, 614, 615, 616,
 619, 620, 621, 622,
 627, 628, 629, 630
 Eagon, R. G., 231
 Eakin, R. E., 425
 Earle, W. R., 606, 607,
 608, 610, 611, 612, 614
 615, 616, 623, 624, 627,
 628, 630
 Eaton, H. D., 449
 Ebashi, F., 481, 489
 Ebashi, S., 481, 487, 488,
 489
 Ebel, J. P., 649
 Eberhart, B. M., 427, 431
 Ebert, M., 181
 Ebisuzake, K., 297, 316
 Ebner, K. E., 612, 623,
 625
 Echols, G. H., 655, 660
 Eckerling, B., 580, 598
 Eckert, B., 184
 Eckert, R. E., 332
 Eddy, A. A., 86
 Eddy, B. E., 658
 Edelhoch, H., 286
 Edelman, J., 348, 353,
 354, 368
 Edlinger, E., 628
 Edman, K. A. P., 489
 Edmonds, M., 170, 175
 Edmundson, A., 109
 Edozien, J. C., 339
 Edsall, J. T., 288
 Edward, J. T., 56
 Edwards, J. E., 395
 Edwin, E. E., 22, 458,
 459, 541
 Eeg Larsen, N., 457
 Egami, F., 568, 643
 Egge, H., 84
 Eggerer, H., 201, 202, 388,
 439, 526, 528, 529, 530,
 531
 Eggleston, L. V., 216
 Ehrenberg, A., 31, 566,
 567
 Ehrenstein, M., 508
 Ehrlich, B., 22
 Eiber, H. E., 63
 Eichel, B., 14
 Eichel, H. J., 23
 Eichhorn, G., 270
 Eichhorn, G. L., 567
 Eichhorn, J., 501, 504
 Eldinoff, M. L., 137, 174,
 617, 618
 Eidus, L. K., 482
 Eiduson, S., 260
 Eigner, E. A., 303
 Eigner, J., 296, 316, 644,
 645, 647, 648
 Eik-Nes, K., 506, 507
 Eirich, F., 560
 Eisen, H. N., 223
 Eisenberg, F., 366
 Eisenberg, H., 309, 316
 Eisengart, A., 460
 Eisenhardt, R., 26
 Eisenstadt, J. M., 373
 Eisenstein, A. B., 404
 Eitigon, M., 372

- Elder, T. D., 231
 El Gholmy, A., 340
 El Hawary, M. F., 340
 Elkjaer, R., 448, 455
 Ellenbogen, E., 477
 Ellenbogen, L., 438
 Elias, E., 364
 Ellington, A. C., 49
 Elliot, W. B., 564
 Elliot, W. H., 252
 Elliott, A., 97, 98
 Elliott, G. H., 475
 Elliott, L. E., 72, 73
 Elliott, W. B., 15, 23
 Elliott, W. H., 207, 396
 Ellis, F., 181, 631
 Ellis, G. P., 45
 Ellis, R. B., 392
 Ellison, S. A., 662
 Ellis, H. A., 24
 Elmore, D. T., 143, 147
 El Nabawy, M., 340
 El Sawi, M. M., 51
 Elsdale, T. R., 174
 Eladen, S. R., 568
 Elsner, V., 631
 Elson, D., 308
 Eltz, R. W., 229, 230
 Elvehjem, C. A., 328
 Elwyn, D., 250
 Ely, J. O., 605
 Emanuel, C. F., 647
 Emerson, G. A., 403
 Emerson, O. H., 257
 Emery, A. J., 663
 Emmanuel, N. M., 141
 Emmart, E. W., 418
 Emmerson, P., 184, 662
 Endahl, B. R., 516
 Endahl, G. L., 516
 Endres, H., 99
 Engel, F. L., 203, 508
 Engel, H. R., 205
 Engel, L. L., 499, 500, 501, 502, 504, 507, 508, 511, 512, 515, 516
 Engelberg, H., 36
 Engelhardt, V. A., 141
 Engelhardt, W. A., 479
 Engeland, A., 448, 455
 Englander, S. W., 184, 662
 Engle, L. L., 244
 Englert, E., 600
 Englesberg, E., 641
 English, R. J., 543
 Engstrom, L., 507
 Ennor, A. H., 489, 251
 Enns, T., 26, 27, 221
 Enrione, M., 168, 170
 Entenman, C., 183
 Entner, N., 165
 Ephrati-Elizur, E., 660
 Ephrussi, B., 625
 Ephrussi-Taylor, H., 117, 295, 661
 Epstein, C., 599
 Epstein, R., 357
 Erb, R. E., 507, 512
 Erbe, S., 447
 Erbland, J., 73, 199, 459
 Erichsen, S., 511
 Erickson, R. E., 542
 Erickson, R. O., 677
 Ericson, L. E., 337
 Ericson, L. G., 100
 Eriksen, L., 557
 Eriksen, N. T., 260
 Ernster, L., 11, 23, 26, 34
 Ershoff, B. H., 449, 462
 Erwin, E. S., 333
 Eschenmoser, A., 533
 Esh, G. C., 449
 Eshelman, L. R., 75
 Espinova, N. G., 99, 100
 Esser, H. O., 464, 542
 Essner, W., 458
 Estabrook, R., 14, 16, 20
 Estabrook, R. W., 27, 28, 564, 565, 567
 Estermann, E. F., 244
 Estes, E. H., Jr., 205
 Etingof, R. N., 611, 615, 625, 629
 Eto, H., 365
 Eugster, C. H., 583
 Evans, C., 384
 Evans, E. A. Jr., 587, 592, 597, 599, 600
 Evans, V. J., 607, 608, 610, 612, 623, 624, 628
 Everett, G. A., 307
 Everett, W. W., 64
 Eyring, E. J., 250
 Ezrin, C., 205

 F
 Faillard, H., 357
 Fain, F. S., 477, 491
 Fakazawa, T., 462
 Falcone, G., 245
 Falk, J. E., 271, 549, 550, 553, 554, 555, 556
 Fandiño, N. G., 75
 Fanshler, D. W., 243, 398
 Faragalla, F. F., 403
 Farber, E., 331
 Farkas, W., 284
 Farley, B., 623
 Farmand, H., 452
 Farmer, D. W. A., 64
 Fasella, P., 261
 Fava, G., 97
 Favarger, P., 207
 Fawcett, J. S., 387
 Fazekas, I. G., 580
 Featherstone, R. M., 625
 Febvre, H., 630
 Fecher, R., 136
 Fedoroff, S., 629
 Feeley, R. J., 329
 Feigelson, P., 30, 34, 246
 Feigen, G. A., 222
 Fein, A., 657
 Feinberg, R. H., 384
 Feindgen, L. E., 680
 Feinendegen, L. E., 172, 174, 617, 621
 Feingold, D. S., 231, 352, 361, 362, 368, 369
 Felaver, W., 481
 Feldheim, W., 459
 Feldman, M. J., 141
 Feldmann, D., 18
 Fell, H. B., 452, 453
 Fellers, C. R., 335
 Fellig, J., 354, 364
 Felsenfeld, G., 117, 118, 119, 595, 596, 647
 Ferguson, J. J., Jr., 526
 Ferguson, R. B., 391
 Fernand, V. S. V., 331
 Fernández, M., 228
 Fernel, W. R., 334
 Ferrar, R. A., 226
 Ferrier, R. J., 45, 64
 Ferrier, W. G., 125
 Fessenden, J. S., 79
 Feur, G., 487
 Feughelman, M., 119
 Fewson, C. A., 240
 Fiala, G., 465
 Fica, A., 296
 Field, E. O., 273, 563
 Field, H., Jr., 207
 Field, R. A., 218
 Fielding, L. L., 508
 Fiertel, A., 87
 Fieser, L. F., 463
 Fliess, H. A., 287
 Figdor, B., 615
 Fiker, S., 71
 Fuller, D. A., 605, 625
 Finan, P. A., 46
 Finch, J. T., 121, 124
 Fincham, J. R. S., 244, 293, 294, 651, 657
 Finck, H., 474, 478
 Findlay, J., 374
 Fink, J., 26, 594
 Fink, K., 138, 462
 Fink, R. M., 138, 462
 Fioramonti, M. C., 607, 608, 610, 612, 627, 628, 630
 Fiori, A., 580
 Firestone, B. Y., 390
 Firth, J. A., 330
 Fischer, E. H., 225, 354, 356, 364, 365, 372, 373
 Fischer, F. G., 580, 581, 583
 Fischer, G. A., 175, 610, 617, 624, 627, 628
 Fischer, H. O. L., 62
 Fish, W. A., 536
 Fisher, E. R., 403, 597
 Fisher, H., 330, 331, 332
 Fisher, H. F., 35, 243
 Fisher, H. W., 608, 610, 611, 628

- Fisher, K. C., 619
 Fisher, K. H., 327
 Fisher, N., 73
 Fisher, R. B., 221, 233
 Fisher, W. D., 184, 643, 677
 Fisher, W. T., 447
 Fishman, J., 511, 512
 Fishman, M., 624
 Fishman, W. H., 359, 368, 516, 595, 600
 Fitch, C. D., 246, 461, 490
 Fitch, W. M., 234
 Fitting, C., 348, 368
 Fitzgerald, G., 404
 Fitzgerald, P. J., 173, 174
 Fitz-James, P. C., 671
 Flaks, J. G., 297, 316, 617
 Flavin, M., 201, 252
 Fleischcher, E. B., 556, 559
 Fleischman, J. B., 646
 Fleischman, R., 607, 612, 613, 620, 628
 Fleming, D. E., 591
 Fleming, L. W., 243
 Fletcher, F., 599
 Fletcher, H. G., 51, 133
 Fletcher, K., 532
 Fling, M., 655
 Flinn, J. H., 540
 Flinn, R. B., 204, 205
 Flodin, N. W., 337
 Flood, A. E., 51
 Flora, A. D., 226
 Florsheim, H. A., 241
 Flory, P. J., 645
 Flowers, M. H., 81
 Floyd, K. W., 176, 177, 631
 Fluaharty, A., 26, 27
 Fluaharty, A. L., 227
 Flute, P. T., 387
 Foley, G. E., 605, 606, 619, 625, 627, 630
 Folkers, K., 22, 48, 56, 437, 478, 526, 532, 533, 542, 543
 Folkers, J. P., 297
 Fomon, S. J., 326, 336
 Fonnesu, A., 26
 Fontanilli, E., 590, 598
 Forbes, R. M., 330
 Forbes, T. R., 507, 512
 Forchielli, E., 509
 Ford, D. K., 627, 630, 631
 Ford, J. E., 335, 437
 Ford, T., 73, 83
 Forrest, H. S., 411, 412, 418, 419
 Forrest, W. W., 275, 558
 Porro, F., 643
 Forsyth, W. G. C., 87
 Foshay, L., 591
 Foss, J. G., 283
 Foster, A. B., 45, 47, 48, 49, 50, 58, 59, 60, 61, 62, 63
 Foster, D. W., 206
 Foster, J. F., 64, 286, 287
 Foster, M., 625
 Fostitropoulos, G., 396
 Fotherby, K., 503, 513
 Fouts, J. R., 366
 Fowler, D. I., 329, 339
 Fowler, K. S., 23
 Fox, E., 662
 Fox, H. C., 339
 Fox, J. J., 133, 134, 136, 137
 Fox, M. S., 659
 Fraenkel, G., 537
 Fraenkel-Conrat, H., 123, 285, 298, 299, 654
 Frame, E. G., 335
 Francis, F. E., 500
 Frandsen, V. A., 512
 Frank, O., 440
 Frankel, F. R., 663
 Franklin, R. E., 121, 122, 123, 124
 Franklyn, T. J., 256
 Franzblau, C., 483
 Franzl, R. E., 199
 Frap, D. L., 452, 453
 Fraser, D., 297, 316, 594, 658
 Fraser, R. D. B., 101
 Frazell, E. L., 207
 Frazier, M. J., 439
 Frederic, J., 678
 Fredrickson, D. S., 189, 190, 203, 204, 205
 Fredericq, P., 659
 Freebairn, H. T., 388
 Freeman, A. E., 607, 608, 614, 616, 630
 Freeman, E. B., 203
 Freeman, H. C., 97
 Freeman, N. K., 74, 189
 Freese, E., 295, 643, 660
 Freifelder, D., 662
 French, D., 351, 355, 358, 371, 372, 373
 French, E. L., 349, 364, 375
 Frenk, S., 338, 340, 341
 Frenster, J. H., 308
 Fresco, J. R., 118, 119, 140, 158, 170, 182, 304, 310, 648, 649, 650
 Frey-Wyssling, A., 126
 Fridovich, I., 36
 Fridovich, J., 284
 Fried, J., 618, 625
 Friedberg, W. R., 205
 Frieden, C., 35, 243
 Friedhoff, A. J., 257
 Friedland, I. M., 397
 Friedman, D. L., 202, 389, 439
 Friedman, H., 165
 Friedman, L., 334, 448, 455, 459
 Friedman, M., 204, 247
 Friedman, M. E., 241
 Friedman, N., 337
 Friedman, P. J., 587
 Friedman, S., 355
 Friedmann, B., 251
 Friedrich, W., 436, 437
 Friend, C., 658
 Frienlander, P. H., 126
 Friesecke, H., 464
 Friesen, B. S., 649
 Friis, J., 217
 Fritz, I. B., 206
 Fritz, J. C., 455
 Frohwein, Y. Z., 65
 Frohwirt, N., 564, 565, 566
 Fromageot, P., 256
 Fromm, H. J., 229
 Frost, D. V., 334, 464, 466
 Frost, P., 32
 Frush, H. L., 64
 Fry, K., 435
 Fryar, A. J., 478
 Fryer, R. I., 543
 Fuchs, F., 489
 Fudge, M., 34
 Fuerst, C. R., 298, 314, 662
 Fuerst, R., 618
 Fujie, Y., 489
 Fujimura, M., 581, 598
 Fujino, Y., 83
 Fujita, A., 454
 Fujiwara, M., 433
 Fukuhara, H., 318
 Fukui, T., 85
 Fukushima, D. K., 503, 513
 Fulton, W. C., 615, 629
 Fuoco, L., 437
 Furberg, S., 114, 115, 125
 Furlong, N. B., 179
 Furminger, I. G. S., 478
 Furth, J., 169
 Furukawa, T., 610, 628
 Furuno, K., 565
 Futterman, S., 228, 426

G

- Gabe, M., 387
 Gabrielyan, N. D., 611
 Gaby, W. L., 85, 259
 Gadebusch, H. H., 46
 Gaffney, T. J., 251, 489
 Gaines, L. M., 624
 Gajdos, A., 549, 551
 Gajdos-Torok, M., 549, 551
 Galbraith, P. S., 396
 Gale, E. F., 297, 318
 Gale, P. H., 22, 543
 Gallagher, C. H., 25, 515
 Gallagher, J., 606
 Gallagher, T. F., 503, 511, 512, 513
 Gallop, P. M., 99, 483
 Galloway, B., 363

- Galvan, R. R., 340, 341
 Ganapathy, S. N., 403
 Gander, J. E., 360
 Ganguly, J., 192, 193, 194
 Garcia, G. J., 336
 Garcia, S., 374
 Gardella, J. W., 182
 Gardiner, R. C., 253
 Gardner, J. N., 76
 Garen, A., 641, 652, 653, 655, 660
 Garfinkel, D., 27, 233
 Garjnobst, L., 427
 Garrett, R. H., 21, 22, 460
 Garrow, J. S., 340
 Gartner, S. M., 622
 Garzillo, M., 433
 Garzo, T., 372
 Gascoigne, J. A., 57
 Gaskill, H., 632
 Gaspar, N. J., 46
 Gasparic, J., 72
 Gastambide-Odier, M., 75
 Gatovskaya, T. V., 100
 Gattuccio, S., 580
 Gaul, C., 508
 Gaul, G., 34, 244, 516
 Gavrilova, L. P., 158, 647, 649
 Gawehn, K., 629
 Gawron, O., 125
 Gay, H., 673, 674
 Gaylor, J. L., 395, 540
 Gebhardt, L. P., 614
 Gelduscheck, E. P., 155, 284, 645, 647, 648
 Geiger, J., 505, 507
 Geiger, P., 195
 Geissler, A. W., 629
 Gelfan, S., 473
 Gelfant, S., 673, 678
 Geller, E., 260
 Gellerman, J. L., 71
 Gellert, M., 490
 Gellert, M. F., 479
 Gemmell, J. P., 203
 Gendre, T., 86
 Genuth, S. M., 300
 George, M., 624
 George, P., 271, 272, 549, 559, 562, 563, 567
 Georgiev, G. P., 141, 649
 Gerald, P. S., 562
 Gerarde, H. W., 623
 Gerbasi, M., 338
 Gerber, G., 254
 Gerber, G. B., 254
 Gerdil, R., 114
 Gergely, J., 475, 476, 478, 479, 487, 488, 489
 Germainier, R., 393
 Gernez-Rieux, C., 33
 Géro, E., 387
 Gerashanovich, V. N., 615, 625, 629
 Gershfeld, M., 483
 Gershoff, S. N., 403
 Gertner, S. B., 258
 Gerulat, B. F., 250, 330
 Gery, I., 580, 587, 588, 589, 599
 Geschwind, I. I., 286, 287
 Geske, G., 483
 Gettler, V. H., 372
 Gewitz, H. S., 563
 Gey, G. O., 609, 611, 614
 Gey, M. K., 609, 611
 Geyer, R. P., 609, 611, 615
 Ghione, M., 355
 Ghosh, A., 393
 Ghosh, J. J., 256, 383
 Ghosh, N. C., 383
 Ghuysen, J. M., 364, 374
 Giardinello, F. E., 619
 Giarman, N. J., 241
 Gibbs, A. J., 124
 Gibbs, M., 535
 Gibbs, R. J., 478
 Gibson, D. M., 192, 195, 202, 389
 Gibson, F., 254, 259
 Gibson, J., 568
 Gibson, J. F., 559
 Gibson, K. D., 550, 551
 Gibson, Q. H., 12, 272, 562, 563
 Gielen, W., 84
 Gierer, A., 7, 121, 298, 299, 661
 Gifford, G. E., 611, 615, 616
 Giglio, E., 115, 116
 Gilbert, C., 336
 Gilbert, G. A., 351
 Gilbert, J. B., 241
 Giles, C. H., 57
 Giles, N. H., 627, 630, 653, 657
 Gilfillan, E. W., 328
 Gilfillan, J. L., 526, 532, 536
 Gilham, P. T., 139, 149
 Gillies, N. E., 566
 Gillman, J., 336
 Gilmour, D., 482
 Gilvarg, C., 239, 252
 Ginsburg, A., 281, 435
 Ginsburg, V., 142, 219, 230, 363
 Ginsburg, Y., 616
 Giraldez, A., 220
 Giri, K. V., 355
 Giroud, C. J. P., 501, 504
 Girre, L., 73
 Gish, D. T., 123
 Gitlin, D., 341
 Giuffrè, N., 618, 619, 625
 Giusti, G., 612
 Gjeddebaek, N. F., 455
 Gjone, E., 73, 81
 Gladner, J. A., 478, 484
 Gladstone, L., 171
 Glahn, P. E., 37
 Glaid, A. J., 125
 Glaser, E., 598
 Glaser, I., 231
 Glaser, L., 361, 362, 364
 Glass, C. A., 78
 Glass, E. A., 216
 Glassman, E., 299, 300, 303, 304, 307
 Glaze, R., 20
 Glazer, A. N., 286, 287
 Glenister, D. W., 509
 Glick, D., 24, 25, 391
 Glinos, E. B., 590
 Gliozzi, M. A., 615
 Gloor, U., 448, 449, 452, 459, 542, 543
 Glover, F. L., 609
 Glover, J., 449, 543
 Glover, S. W., 656
 Glusker, J. P., 125
 Goddard, D. R., 568
 Godman, G., 623
 Godnev, T. N., 553, 555
 Goedde, H. W., 229
 Goedkoop, J. A., 126
 Goetz, G. S., 83
 Gofman, J. W., 189
 Gold, E. M., 509
 Gold, N. I., 511
 Gold, R., 559
 Gold, V., 241
 Goldberg, A., 386, 557
 Goldemberg, S. H., 223, 224, 362
 Golder, R. H., 27, 28
 Goldfarb, D. M., 658
 Goldfarb, R. B., 658
 Goldfein, A., 204
 Goldfine, H., 249
 Goldin, A., 228
 Goldman, D. S., 244
 Goldman, L., 143
 Goldsmith, G. A., 396, 540
 Goldstein, G., 632
 Goldstein, J., 301
 Goldstein, L., 172, 173, 246, 617
 Goldstein, M., 257, 258, 503, 504
 Goldstein, M. N., 606, 632
 Goldstein, M. S., 221
 Goldstein, T. J., 65
 Goldthwait, D. A., 168, 169, 303
 Goldzieher, J. W., 505, 507, 512
 Golenko, Zh. M., 658
 Gomez, F., 338, 340, 341
 Gómez-Sánchez, A., 50
 Gonnard, P., 400
 Gonzales, C., 165
 Gonzales, J. M., 337
 Goodhart, R. S., 402
 Goodman, H. M., 203, 204
 Goodman, I., 366
 Goodman, L., 51, 133, 134
 Goodman, M., 57
 Goodwin, R. F. W., 453

- Goodwin, T. W., 410, 411, 412, 418, 419, 449, 540, 541, 542
 Goodyear, S., 462
 Gopalan, C., 327, 338
 Gopalan, G., 394
 Goransson, A., 207
 Gordon, J., 307
 Gordon, J. E., 338
 Gordon, M. P., 133, 138
 Gordon, M. W., 30
 Gordon, R. S., 462
 Gordon, R. S., Jr., 190, 191, 203, 204, 205, 333
 Gordon, S., 207
 Gorin, P. A. J., 56, 62
 Gorski, J., 507, 512
 Goswami, A., 403
 Goswami, M. N. D., 387
 Gothoskar, B. P., 250, 607
 Gots, J. S., 658
 Gotterer, G. S., 28
 Gottschalk, A., 361, 367, 375
 Gould, A., 14
 Gould, B. S., 623
 Gould, E. A., 606, 617
 Goulet, K. A., 605, 625
 Gounelle, J. C., 456
 Gouvea, M. A., 475, 476, 487
 Grado, C., 391
 Graf, L., 84
 Graff, S., 612, 613, 614, 615
 Graham, A. F., 315, 620, 631
 Graham, D., 329
 Graham, E. R. B., 375
 Graievsky, E. J., 141
 Gram, M. R., 195
 Gramer, F. D., 139
 Gran, F. C., 456, 457
 Grangaud, R., 451, 452
 Granick, S., 272, 550, 551, 552, 553, 554, 555, 556, 557, 567
 Grant, D., 63
 Grant, P. M., 65
 Grant, P. T., 432
 Grant, R. F., 74
 Grassetti, D. R., 243, 398
 Grassl, M., 247
 Grassmann, W., 99
 Gratzner, W. B., 269, 274, 275, 276, 277, 560, 564
 Graves, D. J., 225
 Gray, C. T., 465
 Gray, E. D., 179, 180
 Gray, G. M., 82, 199
 Gray, J. H., 605
 Grazi, E., 226, 232
 Green, A. L., 245
 Green, D. E., 11, 18, 19, 20, 22
 Green, D. W., 93, 102, 103, 104, 105, 116, 560
 Green, J., 22, 458, 459, 541
 Green, M., 615, 629
 Green, N. M., 623
 Green, S., 385
 Greenberg, D. M., 81, 203, 253, 255, 426
 Greenberg, G. R., 297, 316
 Greenberg, L. D., 404
 Greenberg, L. J., 24, 25
 Greenberg, S. M., 537
 Greene, D. E., 331
 Greene, R. C., 581, 585
 Greengard, O., 30, 34, 246, 308
 Greengard, P., 27, 241
 Greenlees, J., 14
 Greenough, J. J., 245
 Greens, J., 359, 368
 Greenstein, J. P., 125, 611
 Greenstein, J. T., 330
 Greenstein, M. E., 330
 Greenwood, C., 12
 Greenwood, C. T., 45
 Greer, S., 662
 Gregg, N. C., 632
 Gregory, K. F., 34, 228
 Greslin, J. G., 536
 Greull, G., 196
 Greville, G. D., 26, 481
 Grey, E. H., 337
 Grey, T. C., 483, 489
 Grey, T. F., 79
 Griffin, A. C., 179
 Griffin, C. E., 79
 Griffith, J. S., 549, 651
 Griffith, W. H., 329
 Grifo, A. P., 449
 Grillo, M. A., 248, 390
 Grimes, A. J., 257
 Griminger, P., 332, 464, 465, 466
 Grinstead, R. R., 386
 Grinstein, M., 557
 Grisolia, S., 228, 247, 248, 491
 Grob, E. C., 540, 541
 Grobstein, C., 625, 631
 Grodsky, G. M., 360
 Gronvall, J. A., 22
 Gros, F., 293, 297, 302, 308, 314, 315, 316
 Gross, A. L., 663
 Gross, D., 302
 Gross, E., 242
 Gross, G., 436
 Gross, J., 100, 641
 Gross, P. R., 672, 676
 Gross, R. T., 34
 Gross, S., 388
 Gross, S. R., 657
 Grosser, B. I., 510, 511, 618
 Grossfeld, H., 615, 619, 623
 Grossil, L. G., 311
 Grossman, L., 175, 649
 Grossowicz, N., 582, 591, 598, 599
 Grove, P. F., 73
 Groves, P. W., 625, 631
 Gruda, J., 484
 Gruell, G., 434
 Grünberger, D., 385
 Grunberg-Manago, M., 119, 158, 166, 167, 304
 Grunze, H., 139
 Grushetskaya, L. A., 79
 Guba, F., 476
 Gubarev, E. M., 87
 Guberniev, M. A., 141
 Gubilevskaya, N. A., 141
 Guerin, M. M., 605
 Guerra, F., 26, 594
 Guest, J. R., 255, 439, 440
 Guggenheim, K., 195, 337
 Guggenheim, M., 597
 Guha, B. C., 337, 383
 Guiditta, A., 24
 Guidotti, G., 561
 Guilbert, P., 326
 Guillory, R. J., 224, 362
 Guinand, M., 86
 Gullekson, E., 260, 516
 Gullis, A. F., 102, 111, 112, 113, 114
 Gundlach, H. G., 278
 Gunja, Z. H., 351
 Gunstone, F. D., 71, 78
 Gunville, R., 512
 Gupta, B. N., 453
 Gupta, J. D., 332
 Gupta, P. S., 341
 Gupta, R. K., 78
 Gupta, S. C., 79
 Gupta, V. D., 101
 Gurevitch, J., 580, 598
 Gurin, S., 208, 501
 Gurnani, S., 201, 439
 Gut, M., 501, 503, 504
 Gutfreund, H., 37
 Guthrie, R., 433
 Guthrie, R. D., 46, 62, 64
 Guthe, K. F., 482
 Gutter, F. J., 276
 Guzman, M. A., 341
 Gwatkin, R. B. L., 609, 610, 628, 631
 Gyorgy, P., 326

H

- Habeeb, A. F. S. A., 276
 Habel, K., 632
 Haber, E., 280
 Habermann, V., 169, 397
 Habermannova, S., 397
 Hadberg, H., 623
 Hadzi, D., 57
 Hafl, R. F., 628
 Hageman, E. C., 612, 625
 Hagen, A. A., 509, 511, 512

- Galvan, R. R., 340, 341
 Ganapathy, S. N., 403
 Gander, J. E., 360
 Ganguly, J., 192, 193, 194
 Garcia, G. J., 336
 Garcia, S., 374
 Gardella, J. W., 182
 Gardiner, R. C., 253
 Gardner, J. N., 76
 Garen, A., 641, 652, 653, 655, 660
 Garfinkel, D., 27, 233
 Garnjobst, L., 427
 Garrett, R. H., 21, 22, 460
 Garrow, J. S., 340
 Gartler, S. M., 622
 Garzillo, M., 433
 Garzo, T., 372
 Gascoigne, J. A., 57
 Gaskill, H., 632
 Gaspar, N. J., 46
 Gasparic, J., 72
 Gastambide-Odier, M., 75
 Gatovskaya, T. V., 100
 Gattuccio, S., 580
 Gaul, C., 508
 Gaull, G., 34, 244, 516
 Gavrilova, L. P., 158, 647, 649
 Gawehn, K., 629
 Gawron, O., 125
 Gay, H., 673, 674
 Gaylor, J. L., 395, 540
 Gebhardt, L. P., 614
 Geldusche, E. P., 155, 284, 645, 647, 648
 Geiger, J., 505, 507
 Geiger, P., 195
 Geissler, A. W., 629
 Gelfan, S., 473
 Gelfant, S., 673, 678
 Geller, E., 260
 Gellerman, J. L., 71
 Gellert, M., 490
 Gellert, M. F., 479
 Gemmell, J. P., 203
 Gendre, T., 86
 Genuth, S. M., 300
 George, M., 624
 George, P., 271, 272, 549, 559, 562, 563, 567
 Georgiev, G. P., 141, 649
 Gerald, P. S., 562
 Gerarde, H. W., 623
 Gerbasl, M., 338
 Gerber, G., 254
 Gerber, G. B., 254
 Gerdil, R., 114
 Gergely, J., 475, 476, 478, 479, 487, 488, 489
 Germanier, R., 393
 Gernez-Rieux, C., 33
 Géro, E., 387
 Gershanovich, V. N., 615, 625, 629
 Gershfeld, M., 483
 Gershoff, S. N., 403
 Gertner, S. B., 258
 Gerulat, B. F., 250, 330
 Gery, I., 580, 587, 588, 589, 599
 Geschwind, I. I., 286, 287
 Geske, G., 483
 Gettler, V. H., 372
 Gewitz, H. S., 563
 Gey, G. O., 609, 611, 614
 Gey, M. K., 609, 611
 Geyer, R. P., 609, 611, 615
 Ghione, M., 355
 Ghosh, A., 393
 Ghosh, J. J., 256, 383
 Ghosh, N. C., 383
 Ghuysen, J. M., 364, 374
 Giardinello, F. E., 619
 Giarman, N. J., 241
 Gibbs, A. J., 124
 Gibbs, M., 535
 Gibbs, R. J., 478
 Gibson, D. M., 192, 195, 202, 389
 Gibson, F., 254, 259
 Gibson, J., 568
 Gibson, J. F., 559
 Gibson, K. D., 550, 551
 Gibson, Q. H., 12, 272, 562, 563
 Gielen, W., 84
 Gierer, A., 7, 121, 298, 299, 661
 Gifford, G. E., 611, 615, 616
 Giglio, E., 115, 116
 Gilbert, C., 336
 Gilbert, G. A., 351
 Gilbert, J. B., 241
 Giles, C. H., 57
 Giles, N. H., 627, 630, 653, 657
 Gilfillan, E. W., 328
 Gilfillan, J. L., 526, 532, 536
 Gilham, P. T., 139, 149
 Gillies, N. E., 566
 Gillman, J., 336
 Gilmour, D., 482
 Gilvarg, C., 239, 252
 Ginsburg, A., 281, 435
 Ginsburg, V., 142, 219, 230, 383
 Ginsburg, Y., 616
 Giraldez, A., 220
 Giri, K. V., 355
 Giroud, C. J. P., 501, 504
 Girre, L., 73
 Gish, D. T., 123
 Gitlin, D., 341
 Giuffre, N., 618, 619, 625
 Giusti, G., 612
 Gjeddlebaek, N. F., 455
 Gjone, E., 73, 81
 Gladner, J. A., 478, 484
 Gladstone, L., 171
 Glahn, P. E., 37
 Glaid, A. J., 125
 Glaser, E., 598
 Glaser, I., 231
 Glaser, L., 361, 362, 364
 Glass, C. A., 78
 Glass, E. A., 216
 Glassman, E., 299, 300, 303, 304, 307
 Glaze, R., 20
 Glazer, A. N., 286, 287
 Glenister, D. W., 509
 Glick, D., 24, 25, 391
 Glinos, E. B., 590
 Gliozzi, M. A., 615
 Gloor, U., 448, 449, 452, 459, 542, 543
 Glover, F. L., 609
 Glover, J., 449, 543
 Glover, S. W., 656
 Glusker, J. P., 125
 Goddard, D. R., 568
 Godman, G., 623
 Godnev, T. N., 553, 555
 Goedde, H. W., 229
 Goedkoop, J. A., 126
 Goetz, G. S., 83
 Gofman, J. W., 189
 Gold, E. M., 509
 Gold, N. I., 511
 Gold, R., 559
 Gold, V., 241
 Goldberg, A., 386, 557
 Goldemberg, S. H., 223, 224, 362
 Golder, R. H., 27, 28
 Goldfarb, D. M., 658
 Goldfarb, R. B., 658
 Goldfein, A., 204
 Goldfine, H., 249
 Goldin, A., 228
 Goldman, D. S., 244
 Goldman, L., 143
 Goldsmith, G. A., 396, 540
 Goldstein, G., 632
 Goldstein, J., 301
 Goldstein, L., 172, 173, 246, 617
 Goldstein, M., 257, 258, 503, 504
 Goldstein, M. N., 606, 632
 Goldstein, M. S., 221
 Goldstein, T. J., 65
 Goldthwait, D. A., 168, 169, 303
 Goldzieher, J. W., 505, 507, 512
 Golenko, Zh. M., 658
 Gomez, F., 338, 340, 341
 Gómez-Sánchez, A., 50
 Gonnard, P., 400
 Gonzales, C., 165
 Gonzales, J. M., 337
 Goodhart, R. S., 402
 Goodman, H. M., 203, 204
 Goodman, I., 366
 Goodman, L., 51, 133, 134
 Goodman, M., 57
 Goodwin, R. F. W., 453

- Goodwin, T. W., 410, 411,
412, 418, 419, 449, 540,
541, 542
- Goodyear, S., 462
- Gopalan, C., 327, 338
- Gopalan, G., 394
- Goransson, A., 207
- Gordon, J., 307
- Gordon, J. E., 338
- Gordon, M. P., 133, 138
- Gordon, M. W., 30
- Gordon, R. S., 462
- Gordon, R. S., Jr., 190,
191, 203, 204, 205, 333
- Gordon, S., 207
- Gorin, P. A. J., 56, 62
- Gorski, J., 507, 512
- Goswami, A., 403
- Goswami, M. N. D., 387
- Gothskar, B. P., 250, 607
- Gots, J. S., 556
- Gotterer, G. S., 28
- Gottschalk, A., 361, 367,
375
- Gould, A., 14
- Gould, B. S., 623
- Gould, E. A., 606, 617
- Goulet, K. A., 605, 625
- Gounelle, J. C., 456
- Gouvea, M. A., 475, 476,
487
- Grado, C., 391
- Graf, L., 84
- Graff, S., 612, 613, 614,
615
- Graham, A. F., 315, 620,
631
- Graham, D., 329
- Graham, E. R. E., 375
- Graievsky, E. J., 141
- Gram, M. R., 195
- Gramer, F. D., 139
- Gran, F. C., 456, 457
- Grangaud, R., 451, 452
- Granick, S., 272, 550, 551,
552, 553, 554, 555, 556,
557, 567
- Grant, D., 63
- Grant, P. M., 65
- Grant, P. T., 432
- Grant, R. F., 74
- Grasseti, D. R., 243, 398
- Grassl, M., 247
- Grassmann, W., 99
- Gratzer, W. B., 269, 274,
275, 276, 277, 560, 564
- Graves, D. J., 225
- Gray, C. T., 465
- Gray, E. D., 179, 180
- Gray, G. M., 82, 199
- Gray, J. H., 605
- Grazi, E., 226, 232
- Green, A. L., 245
- Green, D. E., 11, 18, 19,
20, 22
- Green, D. W., 93, 102, 103,
104, 105, 116, 560
- Green, J., 22, 458, 459,
541
- Green, M., 615, 629
- Green, N. M., 623
- Green, S., 385
- Greenberg, D. M., 81, 203,
253, 255, 426
- Greenberg, G. R., 297, 316
- Greenberg, L. D., 404
- Greenberg, L. J., 24, 25
- Greenberg, S. M., 537
- Greene, D. E., 331
- Greene, R. C., 581, 585
- Greengard, O., 30, 34,
246, 308
- Greengard, P., 27, 241
- Greenlees, J., 14
- Greenough, J. J., 245
- Greens, J., 359, 368
- Greenstein, J. P., 125, 611
- Greenstein, J. T., 330
- Greenstein, M. E., 330
- Greenwood, C., 12
- Greenwood, C. T., 45
- Greer, S., 662
- Gregg, N. C., 632
- Gregory, K. F., 34, 228
- Greslin, J. G., 536
- Greull, G., 196
- Greville, G. D., 26, 481
- Grey, E. H., 337
- Grey, T. C., 483, 489
- Grey, T. F., 79
- Griffin, A. C., 179
- Griffin, C. E., 79
- Griffith, J. S., 549, 651
- Griffith, W. H., 329
- Grifo, A. P., 449
- Grillo, M. A., 248, 390
- Grimes, A. J., 257
- Griminger, P., 332, 464,
465, 466
- Grinstead, R. R., 386
- Grinstein, M., 557
- Grisolia, S., 228, 247, 248,
491
- Grob, E. C., 540, 541
- Grobstein, C., 625, 631
- Grodsky, G. M., 360
- Gronvall, J. A., 22
- Gros, F., 293, 297, 302,
308, 314, 315, 316
- Gross, A. L., 663
- Gross, D., 302
- Gross, E., 242
- Gross, G., 436
- Gross, J., 100, 641
- Gross, P. R., 672, 676
- Gross, R. T., 34
- Gross, S., 388
- Gross, S. R., 657
- Grosser, B. I., 510, 511,
618
- Grossfeld, H., 615, 619,
623
- Grossil, L. G., 311
- Grossman, L., 175, 649
- Grossowicz, N., 582, 591,
598, 599
- Grove, P. F., 73
- Groves, P. W., 625, 631
- Gruda, J., 484
- Gruell, G., 434
- Grünberger, D., 385
- Grunberg-Manago, M., 119,
158, 166, 167, 304
- Grunze, H., 139
- Grushetskaya, L. A., 79
- Guba, F., 476
- Gubarev, E. M., 87
- Guberniev, M. A., 141
- Gubilevskaya, N. A., 141
- Guerin, M. M., 605
- Guerra, F., 26, 594
- Guest, J. R., 255, 439,
440
- Guggenheim, K., 195, 337
- Guggenheim, M., 597
- Guha, B. C., 337, 383
- Guiditta, A., 24
- Guidotti, G., 561
- Guilbert, P., 326
- Guillory, R. J., 224, 362
- Guinand, M., 86
- Gullekson, E., 260, 516
- Gullis, A. F., 102, 111, 112,
113, 114
- Gundlach, H. G., 278
- Gunja, Z. H., 351
- Gunstone, F. D., 71, 78
- Gunville, R., 512
- Gupta, B. N., 453
- Gupta, J. D., 332
- Gupta, P. S., 341
- Gupta, R. K., 78
- Gupta, S. C., 79
- Gupta, V. D., 101
- Gurevitch, J., 580, 598
- Gurin, S., 208, 501
- Gurnani, S., 201, 439
- Gut, M., 501, 503, 504
- Gutfreund, H., 37
- Guthrie, R., 433
- Guthrie, R. D., 46, 62, 64
- Guthe, K. F., 482
- Gutter, F. J., 276
- Guzman, M. A., 341
- Gwatkin, R. B. L., 609,
610, 628, 631
- Gyorgy, P., 326

H

- Habeeb, A. F. S. A., 276
- Habel, K., 632
- Haber, E., 280
- Habermann, V., 169, 397
- Habermannová, S., 397
- Hadberg, H., 623
- Hadzi, D., 57
- Haff, R. F., 628
- Hageman, E. C., 612, 625
- Hagen, A. A., 509, 511,
512

- Hagen, J. H., 205
 Hagen, P., 398
 Hagen, U., 184, 663
 Hagerman, D. D., 34, 221, 244, 511, 516
 Haggroth, S., 55
 Hagihara, B., 13, 26, 364
 Hagihira, H., 253
 Hagopian, M., 513
 Hahn, P. F., 556
 Hahn, T., 96
 Haines, S., 61
 Haining, J. L., 85
 Håjck, V., 71
 Hajra, A. K., 79
 Hakala, M. T., 608, 614, 616, 617, 618
 Hakim, A. A., 165, 277, 278
 Halenz, D. R., 202, 203, 388
 Halevy, S., 615
 Halkerston, I. D. K., 501, 504
 Hall, A. N., 259
 Hall, B. D., 158, 309, 316
 Hall, C. E., 120, 124, 309
 Hall, G. E., 82
 Hall, J. C., 206
 Hall, L. M., 248
 Hall, R. H., 139
 Hall, R. J., 75
 Halla, M., 515
 Hallaway, B. E., 487
 Halle, W., 623
 Hallgren, B., 74
 Halpern, M., 366
 Halpern, Y. S., 246
 Halvorson, H., 245, 356
 364, 370, 620
 Halvorson, H. O., 216, 234, 293, 357
 Ham, R. G., 608
 Hamaguchi, K., 374
 Hamblen, E. C., 508
 Hamilton, J. G., 73, 396, 540
 Hamilton, L. D., 117, 119, 158, 647
 Hamilton, R. D., 252
 Hamm, D., 511
 Hamm, D. L., 510
 Hammaker, L., 360
 Hammet, F. S., 681
 Hamoir, G., 473, 477
 Hamolsky, M., 228
 Hammond, E. G., 75
 Hampton, A., 133, 137, 138
 Hampton, B. L., 75
 Hanahan, D. J., 198
 Hanahan, D. U., 11
 Hanby, W. E., 97
 Hancock, R., 249
 Handler, A. H., 627, 630
 Handler, P., 36, 284, 433, 434
 Handschumacher, R. E., 314, 318
 Handwerk, R. L., 458
 Hane, S., 506
 Hanewald, K. H., 454
 Hanks, D. P., 76
 Hanley, J. B., 448
 Hannah, J., 553
 Hannig, K., 99
 Hansen, J. D. L., 337, 339
 Hansen, P. A., 591
 Hansen, R. P., 72
 Hanson, A. W., 126
 Hanson, J., 474, 475, 485, 486
 Hanson, R., 538, 539
 Happold, F. C., 259
 Hara, I., 87
 Hara, Y., 598
 Harada, M., 245
 Harary, I., 623
 Harbers, E., 168, 183, 617
 Harbury, H. A., 565, 566
 Hardy, F. E., 137
 Hardy, R. W. F., 395, 540
 Harford, C. G., 611, 616
 Harington, C. R., 431
 Harker, D., 102, 104
 Harker, D. H., 277
 Harlan, W. R., Jr., 205
 Harlova, G. V., 141
 Harman, J. W., 474
 Harms, R. H., 466
 Harper, A. E., 328, 331, 332, 333
 Harrap, B. S., 269, 281
 Harrington, H., 181, 631
 Harrington, W. F., 278, 285, 477, 478
 Harris, C. L., 327, 329
 Harris, D. L., 428, 431
 Harris, D. N., 649
 Harris, H., 86, 172, 174, 611, 612, 613, 614, 615, 617, 618, 620, 631
 Harris, J. W., 560
 Harris, P. J., 671
 Harrison, G. A., 580
 Harrison, H. C., 456, 457
 Harrison, H. E., 456, 457
 Harrison, K., 464
 Harrison, P. M., 95, 97, 102
 Hart, R. G., 102, 106, 108, 109, 122, 270, 271, 559, 560
 Harte, E. M., 256
 Hartiel, W., 455
 Hartigan, J., 349
 Hartman, L., 75
 Hartman, P. E., 656, 658
 Hartman, S. C., 165
 Hartman, Z., 656, 658
 Hartmann, G., 299, 300, 301, 307, 311, 312
 Hartree, E. F., 12, 13, 82, 564, 565, 568
 Hartshorne, D. J., 486
 Hartsook, E. W., 327
 Harvey, E. B., 669
 Harvey, J. D., 449
 Harwood, H. J., 79
 Hase, E., 681, 682
 Hasegawa, H., 18, 19
 Hasegawa, K., 598
 Hasegawa, S., 581, 598
 Haselkorn, R., 158, 304, 310, 648, 649
 Hash, J. H., 357
 Hashim, S. A., 204
 Hashimoto, Y., 357, 482
 Haskell, J., 503, 505, 512
 Hass, K. B., 45
 Hass, L. F., 231
 Hasselbach, W., 480, 481, 489
 Hasserodt, U., 273, 274, 562, 563
 Hassid, W. Z., 231, 348, 359, 361, 362, 363, 365, 367, 369
 Hastings, A. B., 233
 Hatch, F. T., 255, 440
 Hatell, Y., 22, 426, 441
 Hatt, H. H., 77
 Hattori, K., 610, 611, 614
 Hauck, F. P., 553
 Hauenstein, J. D., 284
 Haug, A., 561, 563
 Hawk, R., 362
 Haupt, I., 34
 Hausberger, F. X., 192
 Hauschka, T. S., 627
 Hause, N. L., 335
 Havel, R. J., 204
 Havskov Sørensen P., 449
 Hawes, R., 586, 587
 Hawke, J. C., 72, 75
 Hawkins, G. R., 175
 Hawkins, N. M., 612
 Hawkinson, V. E., 551
 Haworth, W. N., 351
 Hawthorne, J. N., 198, 392, 394
 Hawtreay, A. O., 27, 28
 Hayaishi, O., 260
 Hayashi, H., 253
 Hayashi, K., 374
 Hayashi, M., 597
 Hayashi, T., 476, 479
 Hayes, T. L., 189
 Hayes, W., 641
 Haylick, L., 631
 Haynes, R. C., 138
 Hays, V. W., 452, 453
 Hayward, A. C., 87
 Hazelwood, O. M., 258, 386
 Head, F. S. H., 62
 Healy, G. M., 620
 Hearn, H. J., 631
 Hearn, W. R., 503
 Heath, E. C., 142, 435
 Heath, H., 550, 551, 554, 555
 Hebb, C. R., 619

- Hebblethwaite, G., 66
 Hecht, L. I., 167, 169, 299, 300, 301, 302, 303, 310, 315
 Hechter, O., 501, 504
 Hedbom, A., 438
 Hedén, C. G., 8
 Hedgley, E. J., 45, 51
 Hedrick, J. L., 251
 Hegre, C. A., 203
 Hegsted, D. M., 326
 Hehre, E. J., 347, 349, 350, 352, 356
 Heicken, K., 5
 Heicklin, L., 73
 Heidelberg, C., 168, 183, 617
 Heidelberg, M., 45
 Heikel, T., 557
 Heilbroun, E., 400
 Heilbrunn, L. V., 678
 Heim, W. G., 33
 Heinz, E., 241
 Heinz, F., 230
 Heinzelman, R. V., 261
 Hejnova, L., 216, 217, 218
 Helander, E., 473
 Hell, E., 181
 Hellebust, D. M., 387
 Hellesner, C. W., 255, 439, 440
 Hellerman, L., 24
 Hellig, H., 532
 Hellman, L., 207
 Hellman, N. N., 349
 Helmreich, E., 223
 Helmut, J. H., 79
 Hemming, F. W., 542
 Hems, G., 184, 662
 Hems, R., 24
 Henbest, H. B., 60, 61
 Hendee, E. D., 619
 Henderson, L. M., 394
 Henderson, M. J., 218, 221
 Henderson, R. W., 19, 565
 Hendler, R. W., 85
 Hendlin, D., 22
 Henglein, F. A., 45, 57
 Henington, V. M., 338
 Henle, G., 615, 629
 Henneman, D. H., 203, 204
 Henneman, P. H., 203, 204
 Henning, U., 199, 526, 528, 529, 530, 531
 Henrotte, J. G., 473
 Heppel, L. A., 157, 166, 167, 650
 Herbert, E., 167, 168, 169, 170, 301, 303, 304, 315
 Herbert, V., 440
 Herbst, A. L., 509
 Herbst, E. J., 580, 581, 582, 587, 588, 589, 590, 593, 594, 598, 599
 Herčfk, F., 8
 Heredia, C. F., 216, 228, 240
 Herissey, H., 372
 Hermans, J., 271, 283, 645, 648
 Hernandez, A., 223
 Hernandez, H. J., 449
 Herndon, J. F., 537
 Herr, E. B., 484
 Herranen, A. M., 516
 Herriott, R. M., 4
 Hers, H. G., 219, 221, 229, 230
 Hershaberger, T. V., 327
 Hershey, A. D., 315, 316, 317, 583, 646, 647
 Herskovits, T. T., 287
 Hertz, R., 623
 Herzenberg, L. A., 215, 608, 611, 627, 656
 Hess, B., 27, 28, 233
 Hesse, G., 48
 Hesse, R. H., 510
 Hestrin, S., 352, 353, 365, 368
 Hettig, R. A., 556
 Hevesy, G., 180
 Hew, H. Y. C., 663
 Hewson, K., 133
 Heyl, D., 437
 Heyningen, R. van, 229
 Heyns, K., 49, 50
 Heyworth, R., 374
 Hiatt, C. W., 663
 Hiatt, H. H., 175, 233, 384, 616
 Hiatt, R. B., 366
 Hibino, M., 610, 611, 614
 Hickman, H. M., 73
 Higa, S., 241
 Higashi, T., 17, 33, 568
 Higashigawa, M., 87
 Higgins, G. M. C., 543
 Higgins, J., 27, 233
 Highberger, J. H., 100
 Highley, D. R., 438
 Higuchi, K., 610
 Hilbert, G. E., 46
 Hill, A. V., 558
 Hill, D. C., 449
 Hill, D. K., 474
 Hill, R., 557, 568
 Hill, R. B., 620
 Hill, R. J., 275, 558, 561
 Hill, R. L., 557, 560, 562
 Hiller, B., 489
 Hillman, B. R., 218
 Hillman, R. S. L., 241, 403
 Hilmoe, R. J., 166, 167
 Hilschmann, N., 111, 275, 561
 Hilse, K., 275, 561
 Himmelfarb, S., 486
 Hinson, K. A., 372
 Hinze, G., 79
 Hioco, D., 433
 Hirae, H., 33
 Hirano, T., 87
 Hird, F. J. R., 200, 248
 Hirokawa, S., 95, 126
 Hirota, Y., 680
 Hirs, C. H. W., 102, 269, 277
 Hirsch, J. G., 586, 587, 599
 Hirsch, M. L., 302
 Hirsch, R. L., 203
 Hirschmann, F. B., 513
 Hirschmann, H., 513
 Hirst, E. L., 45
 Hisaw, F. L., Jr., 507
 Hitchings, G. H., 427, 366
 Hjarde, W., 447, 449, 450, 453, 454
 Hoaglund, M. B., 169, 293, 298, 299, 300, 301, 303, 304, 308, 309, 310, 311, 312
 Hoare, D. S., 550, 551, 554, 555
 Hobbs, F. L., 612
 Hobbs, G. L., 627, 630
 Hoberman, H. D., 233
 Hobkirk, R., 513
 Hoch, G. E., 239
 Hoch-Ligeti, C., 623
 Hodges, R., 136
 Hodgkin, D. C., 94, 106, 126, 437
 Hoefnagel, M. A., 81
 Hoff, R. F., 608, 627
 Hoffer, A., 540
 Hoffer, M., 133
 Hoffman, C. A., 349
 Hoffman, C. H., 533
 Hoffman, P., 373, 374
 Hoffmann, G., 79
 Hoffmann-Berling, H., 676, 683
 Hofmann, F. G., 502, 505
 Hofmann, K., 75
 Hogan, R., 441
 Hogeboom, G. H., 182
 Hogenkamp, H. P. C., 438, 439
 Hogg, J. F., 219, 228
 Hogness, D. S., 658, 660
 Hohlweg, W., 453
 Hokin, L. E., 81, 196, 197
 Hokin, M. R., 81, 197
 Holden, H. F., 557
 Holdsworth, E. S., 437, 439
 Holiday, E. R., 284
 Holland, J. F., 433
 Hollander, N., 503, 507, 516, 517
 Hollander, V. P., 503, 507, 516, 517
 Hollander, W., 536
 Holleman, J. W., 566
 Hollenberg, C. H., 191, 206
 Holley, R. W., 140, 299, 300, 301, 307
 Holliday, M. E., 514

- Hollingworth, B. R., 286,
308, 309
Hollocher, T., 27, 28, 564,
565
Hollocher, T. C., 24
Holloway, B. W., 296
Hollunger, G., 26
Holly, F. W., 48, 56
Holman, H., 333
Holman, R. T., 76
Holme, D., 76
Holmes, B. E., 182, 183
Holmes, E. G., 333
Holmes, K. C., 101, 121,
122, 123
Holmes, R., 610, 628
Holmes, W. N., 501, 506
Holmstrom, E. G., 503,
505, 512
Holt, A., 63
Holt, L. E., Jr., 326,
329, 339
Holt, N. B., 64
Holtcamp, D. E., 536
Holter, H., 677
Holton, F. A., 20
Holter, A., 477, 478, 479,
648
Holtzer, H., 474, 478, 623,
626, 630, 683
Holzel, A., 220, 231
Holzer, H., 229, 233
Hölzl, J., 391
Homann, J., 612
Honda, Y., 372
Honeyman, J., 62
Hood, B., 207
Hooglaaslan, A. C., 632
Hoogsteen, K., 116, 118
Hooper, C. W., 117, 119,
158, 647
Hooper, J. L., 618, 627
Hoover, C. R., 612, 625
Hopkins, A., 618
Hopkins, J. W., 308
Hopkins, C. Y., 75, 76, 77,
78
Hoppe, W., 126
Hordvik, A., 125
Horeau, A., 79
Horecker, B. L., 215, 216,
229, 230, 232, 435, 616,
622
Hörhammer, L., 73, 74,
78, 391
Hori, M., 615
Horibata, K., 260, 624
Horio, T., 13, 17, 564, 566,
568
Horn, P., 115
Horne, R. W., 122, 124
Horner, A. A., 452
Hornbrook, J. W., 632
Horning, E. C., 455
Horning, M., 531, 532
Horning, M. G., 193, 534
Hornstein, I., 72, 73
Horonick, A., 402
Horowitz, N. H., 250, 655
Horton, A. A., 410
Horton, C. L., 607
Horton, D., 45, 47, 50, 61
Horvath, B., 485
Horváth, Z., 584
Hosaka, S., 610
Hosoya, T., 31
Hotchkiss, J., 242
Hotchkiss, R. D., 641, 659,
660
Hotta, K., 87, 475, 476, 482
Hough, L., 45, 47, 62
Houghton, T. M., 245
Howard, A., 181
Howard, A. N., 387
Howard, B. H., 373
Howard, H. W., 337
Howard, R. B., 27, 29
Howard, R. L., 34
Howe, E. E., 328
Howell, J. McC., 12
Hsia, D. Y., 246
Hsia, S. L., 207
Hsu, C., 246
Hsu, K., 485, 486
Hsu, T. C., 609, 618, 627,
628, 630, 631
Hu, A. S. L., 357, 358, 364
Huang, L., 246
Huang, S., 595, 596
Hubbard, N., 557
Hubbard, R., 451
Hubby, J. L., 420
Hübener, H. J., 512
Huber, G., 56
Huber, M., 115
Hübscher, G., 81, 190, 197
Hudson, B., 256
Hudson, P. B., 500, 504,
506
Hudson, R. V., 619
Huebner, C. F., 47, 48
Huennekens, F. M., 441,
425, 426
Huff, J. W., 526, 532, 536
Huggard, A. J., 61
Huggett, A. St. G., 221
Huggins, C., 205
Huggins, M. L., 99, 100
Hughes, A. F. W., 679, 682
Hughes, B. P., 334
Hughes, E. W., 97
Hugot, D., 456, 457
Huisman, T. H. J., 560
Hulcher, F. H., 349
Hulliger, L., 623
Hülsmann, W. C., 15, 23,
299, 300, 301, 307, 311, 312
Hultin, T., 169, 317
Hummel, J. P., 282
Humpheys, S. R., 228
Hundley, J. M., 335
Hung, M. M., 244
Hünig, S., 79
Hunt, J. A., 274, 275, 294,
561, 562, 653
Hunter, F. E., Jr., 26,
594
Hunter, L., 57
Hunter, W. S., 658
Hurlbert, R. B., 175
Hurlock, B., 509, 510, 515
Hurst, R. O., 513
Hurwitz, A., 26, 594
Hurwitz, J., 169, 171, 435
Huseby, R. A., 505, 507
Hutchinson, D. W., 146
Hutchinson, F., 662
Hutchinson, J. B., 337
Hutchinson, W. D., 274,
314, 563, 564
Hutner, S. H., 437, 440
Hutson, D. H., 356
Huxley, A. R., 488
Huxley, H. C., 120
Huxley, H. E., 122, 124,
309, 474, 485, 486
Huzino, A., 302
Hvidt, A., 281
Hyatt, M., 490
Hyde, W. L., 683
- I
- Iball, J., 126
Ichihara, A., 253
Ichihara, E. A., 253
Ichihara, K., 402
Idler, D. R., 506
Igo, R. P., 11
Iijima, T., 660
Itaka, Y., 95
Ikawa, M., 66, 401
Ikeda, K., 435
Ikenaka, T., 372
Illingworth, B., 224, 226,
362, 364
Iushenko, B. N., 658
Imahori, K., 282, 288
Imai, K., 355, 413, 414,
415, 416, 418, 419
Imai, N., 475, 476
Imai, Y., 258
Imamoto, F., 27, 28
Immers, J., 676
Inagaki, M., 35
Indira, K., 337
Infantellina, F., 681
Ingles, D. L., 63
Ingraham, L. L., 435
Ingram, D. J. E., 109, 110,
258, 559
Ingram, V. M., 103, 105,
111, 275, 276, 277, 294,
558, 560, 561, 562, 653,
654
Inman, R. B., 647
Inoue, S., 677, 663
Inscoc, J., 360
Insull, W., 72
Intengan, C. L., 337
Intieri, O. M., 133

Inui, S., 87
 Iodice, A. A., 551
 Ippen, H., 551
 Irreverre, F., 253
 Irvine, E., 607, 628
 Irving, J. T., 457
 Irzykiewicz, H., 256
 Isbell, H. S., 46, 56, 64
 Isemura, T., 285, 365
 Isherwood, F. A., 383
 Ishii, T., 598
 Ishikura, H., 564, 565, 566
 Ishimoto, M., 566
 Isler, O., 463, 464, 542
 Ismail, S., 340
 Isomoto, A., 37
 Isselbacher, K. J., 190, 360, 515
 Itagaki, E., 240
 Itano, H. A., 273, 294, 314, 560, 561, 562, 563, 564
 Ito, E., 142, 610
 Ito, N., 491
 Ito, S., 553
 Itzhaki, S., 176
 Ivanov, I., 477, 479, 482, 486
 Ivanova, K. I., 400
 Ivanova, T. L., 482
 Iwasa, K., 27
 Iwatsu, T., 433
 Iwatsubo, M., 34
 Iyengar, R., 477
 Izak, G., 192
 Izumiya, N., 125

J

Jackson, A. H., 552, 555
 Jackson, E. L., 584
 Jackson, F. L., 19
 Jackson, P. W., 618, 619, 625
 Jackson, R. W., 349
 Jackson, S. F., 623
 Jacob, F., 234, 297, 298, 314, 316, 643, 653, 655, 656, 659, 660
 Jacob, M., 275, 563
 Jacob, M. I., 490, 491
 Jacobelli, G., 360
 Jacobs, E. E., 27, 28, 34, 567
 Jacobs, F. A., 241, 403
 Jacobs, J., 375
 Jacobson, H. I., 518
 Jacobson, R. A., 125
 Jacobson, W., 674
 Jacques, P., 34
 Jacques, J. A., 682
 Jacquot, R., 341
 Jaenicke, L., 425
 Jaffe, H., 567
 Jahnz, M., 612, 613
 Jain, S. K., 57
 Jakoby, W. B., 243
 Jakovljevic, J. M., 447
 James, A. L., 142, 363
 James, A. T., 72, 77, 78, 190
 James, S. P., 256
 James, T. W., 682
 James, W. O., 568
 Jamieson, G. A., 255, 586
 Janeway, C. A., 341
 Jänicke, S., 73
 Janion, C., 154
 Janka, R., 583
 Janot, M., 584
 Janson, J., 56
 Jantzen, F., 75
 Januszka, J. P., 536
 Jarabak, J., 35
 Jarnefelt, J., 19, 20
 Jarnum, S., 333
 Jasinski, B., 385
 Jast, A., 72
 Jatel, T. P., 141
 Jayle, M. F., 374, 514
 Jaymond, P., 51
 Jeanrenaud, B., 222
 Jebson, J. W., 473
 Jefferies, P. R., 58
 Jeffrey, G. M., 595, 600
 Jeffrey, G. A., 125, 126
 Jeger, O., 533
 Jellinek, F., 126
 Jen, M. H., 485, 486
 Jen, M.-T., 484
 Jencks, W. P., 490
 Jenkins, W. T., 243, 398
 Jensen, E. V., 518
 Jensen, F. C., 624
 Jensen, H., 74
 Jensen, L. H., 95, 97, 115
 Jensen, P. K., 514
 Jennings, A. R., 453
 Jentoft, V. L., 611
 Jepsen, J. B., 259
 Jermolajeva, L. P., 141
 Jermy, M. A., 356, 357, 366, 369
 Jervell, K. F., 516
 Jeske, H. A., 466
 Jeuniaux, O., 374
 Jevreinova, T. N., 141
 Jewell, P. I., 599
 Joel, P. B., 516
 Johns, E. W., 663
 Johns, R. G. S., 86
 Johnson, A. W., 435, 437, 553
 Johnson, B. C., 169, 201, 330, 331, 439, 449, 451, 452, 453, 460, 462, 465
 Johnson, D. S., 508
 Johnson, E., 231
 Johnson, J. A., 133
 Johnson, J. E., 219, 230
 Johnson, L., 415
 Johnson, M. J., 142
 Johnson, P., 477
 Johnson, R. C., 248
 Johnson, W. H., 475, 485
 Johnson, W. S., 64
 Johnston, J. M., 190
 Johnston, N. C., 76
 Johnston, P. V., 72
 Jókay, L., 478
 Jolles, E., 374
 Jollès, P., 86
 Jolles-Bergeret, B., 405
 Jolley, R. L., 246
 Joly, L. P., 258
 Jonas, H., 516
 Jones, D. N., 74, 82
 Jones, E. E., 207, 502
 Jones, E. R. H., 76
 Jones, F., 329
 Jones, F. T., 257
 Jones, G., 373
 Jones, I. C., 501, 507
 Jones, J. K. N., 47, 49, 51, 63
 Jones, K. M., 255, 396, 397
 Jones, M., 537, 623, 625
 Jones, M. E., 247
 Jones, N. R., 490, 491
 Jones, O. T. G., 240, 410, 412
 Jones, R. T., 274, 275, 561, 562, 563, 564
 Jones, S. W., 448, 454, 455
 Jones, W. H., 437
 Jonsen, J., 51
 Jonxis, J. H. P., 277, 560, 561, 566
 Joze, M., 122
 Jordan, D. O., 647
 Jordan, E., 231
 Jordan, H. V., 620
 Joska, S., 611, 614
 Joseph, D., 327
 Joseph, J. P., 134
 Joseph, K., 337
 Joseph, S., 678
 Josse, J., 178, 179, 295, 297, 316, 363, 642, 643
 Jouan, P., 73
 Judah, J. D., 26, 29
 Juhász, E., 597
 Jukes, D. E., 115
 Julietti, F. J., 79
 Julita, P., 437
 Jungas, R. L., 204, 222
 Junk, R., 512
 Juriwara, H. S., 74
 Jütting, G., 195, 202, 388, 389

K

Kabat, E. A., 373
 Kafiani, K. A., 479
 Kagawa, Y., 557, 607
 Kahana, S. E., 227
 Kahlbrock, M., 475
 Kahn, J. S., 475, 485
 Kahn, R. H., 606, 624

- Kaihara, M., 261
 Kaiser, A. D., 295, 658, 660
 Kakinchi, K., 365
 Kakol, I., 484
 Kalas, J. P., 204
 Kalckar, H. M., 216, 231, 294, 348, 359, 366, 612, 615, 628
 Kaldor, G., 487, 488, 489
 Kalif, G. F., 355
 Kalinsky, H., 402
 Kalmanson, A. E., 141
 Kalnitsky, G., 280, 282, 283
 Kalyankar, G. D., 490
 Kamen, M. D., 556, 568
 Kameyama, T., 297
 Kamper, J., 94
 Kandutsch, A. A., 535
 Kaneko, K., 607
 Kanfer, J., 388
 Kantor, T. G., 63
 Kapeller-Adler, R., 254, 587
 Kaplan, A. S., 617, 619
 Kaplan, E., 206
 Kaplan, L., 136
 Kaplan, N. O., 24, 25, 34, 144, 228, 515
 Kar, N. C., 383
 Karasek, M., 300
 Karau, W., 144
 Kardewitz, F., 295
 Kare, M. R., 474
 Kariya, T., 536
 Karmarkar, S. S., 24
 Karmen, A., 190, 193
 Kartha, A. R. S., 458
 Kartha, G., 126
 Karusch, F., 296
 Karvonen, M. J., 221
 Kasai, M., 475
 Kasparow, M., 500, 506
 Kassanis, B., 658
 Kaszka, E. A., 437
 Katagiri, H., 355, 413, 414, 416, 418, 419
 Kates, M., 73
 Kato, I., 619
 Kato, M., 87
 Katoh, S., 568
 Katsuda, H., 607, 610, 611, 614, 615, 627, 628, 630
 Katunuma, N., 420, 422, 423, 424, 425
 Katz, E., 247
 Katz, J., 229
 Katz, J. L., 96
 Katzman, P. A., 501
 Kaufman, B. T., 24, 25
 Kaufman, E., 663
 Kaufman, H. P., 71, 73, 79
 Kaufman, S., 257
 Kaufmann, B. P., 673, 674
 Kaufmann, W., 287, 295
 Kawada, I., 610, 628
 Kawade, Y., 649
 Kawahara, F. S., 514
 Kawamura, N., 676, 682
 Kawana, M., 610, 627, 628
 Kawasaki, H., 433
 Kawasaki, T., 428, 429, 431, 433
 Kawashima, H., 475
 Kay, C. M., 285, 484, 485
 Kay, D., 583, 592, 598
 Kay, L. D., 426, 441
 Kaye, A., 169
 Kazenko, A., 349
 Kazirow, Y., 202, 433
 Kazuno, T., 207
 Kean, E. A., 333
 Kean, E. L., 75
 Kearney, E. B., 24
 Keck, E., 327
 Keck, K., 297, 316
 Keech, D. B., 233, 389
 Keeler, M. M., 484
 Kell, K. D., 64
 Kellin, D., 12, 13, 18, 24, 271, 559, 560, 564, 565, 568
 Kellin, J., 272
 Keir, H. M., 179
 Keise, M., 13
 Keister, D. L., 580, 581, 582, 590, 593, 595, 596, 598, 600
 Kekwick, R. G. O., 543
 Kellenberger, E., 643
 Keller, D. L., 233
 Keller, E. B., 297, 300
 Keller, W., 609
 Kelley, M. B., 333
 Kellie, A. E., 514
 Kellner, A., 203
 Kellner, G., 615, 624
 Kellner, J. D., 402
 Kellock, T. D., 77, 78
 Kellogg, D. S., Jr., 609, 628
 Kelly, L. S., 181, 183
 Kelly, S., 384
 Kemmerer, H. A., 449
 Kemp, P., 392
 Kendall, F. E., 205
 Kendrew, J. C., 93, 94, 96, 97, 102, 104, 105, 106, 107, 108, 109, 110, 118, 119, 269, 270, 271, 559, 560
 Kenkare, V. W., 426
 Kennedy, B., 338
 Kennedy, E. P., 190, 196, 197, 198, 360, 392
 Kenner, G. W., 143, 147
 Kenney, F. T., 246
 Kennison, R. D., 23, 465
 Kenny, G. E., 631
 Kensler, C. J., 246
 Kent, H. N., 611, 614
 Kent, P. W., 64, 65, 66, 361, 374
 Kenten, R. H., 580, 587
 Kepes, A., 215
 Keppeler, J. G., 72, 79
 Keresztesy, J. C., 425
 Kerr, H. A., 612
 Kerr, R. W., 371
 Kersten, H., 502
 Kersten, W., 502
 Keser, M., 358, 364
 Kessel, I., 526, 528, 531
 Kessen, G., 73
 Khan, M. A., 72
 Khin Maung, 351
 Khorana, H. G., 119, 134, 139, 140, 141, 143, 144, 147, 149
 Khorana, H. L., 641
 Khyrn, J. X., 47
 Kibrick, A. C., 71, 254
 Kielley, W. W., 477
 Kies, M. W., 253, 254
 Kiese, M., 563
 Kiesow, L., 216
 Kiess, A. A., 79
 Kihara, H., 241, 591, 598
 Kiknadze, I. I., 141
 Kikuchi, G., 550
 Kilksen, R., 122
 Killip, J. D., 248
 Kilsheimer, G. S., 237
 Kim, J. C., 358
 Kimball, R. F., 679
 Kimura, H., 554
 Kimura, T., 27, 28
 King, D. W., 606, 620
 King, H. K., 243
 King, K. W., 349, 357
 King, M. E., 177
 King, M. V., 102, 103, 105, 282
 King, R. P., 327
 King, T. E., 18, 24, 34, 229, 230
 Kingdon, H. S., 300
 Kinley, L. J., 452
 Kinoshita, J. H., 228
 Kinsella, R. A., Jr., 500
 Kipnis, D. M., 221, 242
 Kirby, G. W., 143
 Kirby, K. S., 140, 649
 Kircher, H., 45
 Kirk, P. L., 177
 Kirkman, H. N., 34, 142, 232, 653
 Kirkwood, S., 584
 Kirschfeld, S., 176
 Kirschner, L. B., 663
 Kishi, T., 413, 414, 415, 418, 419, 420
 Kishimoto, Y., 72, 74
 Kisliuk, R. L., 255, 439, 440
 Kissman, H. M., 133, 134
 Kit, S., 643, 663
 Kitabchi, A. E., 384
 Kitagawa, S., 479, 480, 482, 490
 Kitai, R., 102, 277

- Kitamura, I., 630
 Kitaura, T., 87
 Kito, Y., 451
 Kiyasu, J. Y., 190, 198, 199
 Kjaer, A., 45
 Klain, G. J., 331
 Klatt, O., 627, 630, 631
 Klatzo, I., 485
 Klavins, M., 517
 Klebanoff, S. J., 32
 Klee, W. A., 303
 Kleiber, M., 230, 380
 Klein, E., 627
 Klein, G., 627
 Klein, H. P., 87, 373
 Klein, J. R., 37, 245, 557
 Klein, L., 99
 Kleinfeld, R., 674
 Kleinspehn, G. C., 551, 553
 Kleinzeller, A., 216, 217
 Klemperer, E., 270, 282
 Klenk, E., 76, 77, 78, 84, 375
 Klenow, H., 180
 Kleschick, A., 73
 Klethi, J., 24
 Klimczak, Z., 455
 Klimov, V. Yu., 141
 Kline, I., 619, 627, 629
 Kline, O. L., 454, 455, 459
 Kling, A., 419
 Klingenberg, M., 24, 25, 233
 Klotz, I. M., 287
 Kluwen, H. M., 183
 Klug, A., 94, 121, 122, 123, 124
 Klungsöyr, S., 361
 Klybas, V., 227
 Knapp, A., 404
 Knapp, J., 663
 Knappe, J., 195, 202, 388, 389, 526, 528
 Knapstein, H., 449
 Knauss, H. J., 525, 526
 Knigge, K. M., 502, 504, 506
 Knight, B. C., 553, 555
 Knight, C. A., 123, 642
 Knight, S. G., 229, 230
 Knipprath, W., 77
 Knobil, E., 204, 242
 Knoll, J. E., 137, 174, 617
 Knoller, M. I., 491
 Knox, W. E., 246, 257, 387, 515
 Knuppen, R., 510, 511, 512, 513
 Kny, H., 253
 Kobata, A., 428, 431
 Kobayashi, T., 85, 356, 454
 Koch, G., 317, 658
 Koch, K. F., 78
 Kochakian, C. D., 510, 511, 516
 Kochen, J., 22, 459
 Kodicek, E., 394, 455, 456
 Kodja, A., 259
 Koenig, S., 658
 Koeppe, R. E., 251
 Koepsell, H. J., 349
 Koerner, J. F., 297, 316
 Koffler, H., 85
 Kofler, M., 463, 542
 Kofoed Nielsen, G., 462
 Kogl, F., 126
 Kogut, M., 326
 Kohler, H., 475, 479, 510
 Kohn, P., 63
 Kohtes, L., 354, 364
 Koike, M., 35, 36
 Koizumi, I., 449
 Kok, I. B., 141
 Kolachov, P., 355
 Kolb, E., 449
 Kominz, D. R., 477, 484, 485, 486
 Komoda, T., 583
 Kon, S. K., 394, 437, 448
 Kondo, N., 663
 Konieczny, L., 560
 Konig, T., 477
 Konigsberg, W., 275, 561
 Koningsberger, V. V., 301, 318
 Konishi, K., 482
 Kono, T., 252
 Konogi, H., 165
 Konuisky, F. R., 22
 Kopac, M. J., 254
 Kopilov, V. A., 141
 Kopin, I. J., 258, 261
 Koptelova, E. I., 141
 Koransky, W., 139
 Koritz, S. B., 138, 296
 Kormendy, K., 584
 Korn, E. D., 191, 373, 374
 Korn, M., 305
 Kornberg, A., 6., 141, 177, 178, 179, 182, 196, 295, 297, 316, 363, 611, 616, 642, 643, 650
 Kornberg, S. R., 297, 316, 642
 Kornfeld, R., 226
 Kornfeld, S., 231, 362, 364
 Korngold, G. C., 140, 180, 182
 Korolev, N. V., 141
 Korte, F., 411, 414, 420, 421, 423, 425, 430, 431
 Korytnyk, W., 45
 Kosaki, G., 610
 Kōsaki, T., 581, 598
 Koser, S. A., 390
 Koshland, D. E., Jr., 348, 366, 369, 370, 482, 484
 Kost, A. N., 584
 Kösters, B., 45
 Kostyo, J. L., 242
 Koszalka, T. R., 473
 Kotani, S., 87
 Kotani, T., 581, 598
 Kotani, Y., 581
 Koteles, G., 476
 Kotyk, A., 216
 Kouhoup, R., 79
 Kovacs, A., 597
 Kovacs, F., 24
 Kovács, T., 477, 478
 Kövér, A., 477, 478
 Kowalsky, A. G., 227
 Koyama, J., 372, 568
 Koyama, Y., 598
 Koyanagi, T., 453
 Krah, M. E., 679
 Kram, D. C., 246
 Kramer, J. H. van de, 220
 Krampitz, L. O., 434
 Krane, S. M., 219, 220
 Kranz, Z. H., 73
 Krasna, A. I., 436
 Kratzer, F. H., 253
 Kraus, H., 679
 Krause, E.-G., 490
 Krause, M., 174
 Krause, R. F., 452
 Kraut, J., 102, 115
 Kraychy, S., 512
 Krebs, E. G., 225, 364
 Krebs, H. A., 200, 216, 249
 Krichevskaya, A. A., 615
 Krieg, D. R., 662
 Krimm, S., 101
 Kring, P. L., 449
 Krisch, K., 16, 17
 Krishna Murti, C. R., 20
 Krishnamurthy, K., 337
 Krishnaswamy, P. R., 249, 300, 302
 Kristen, H., 51
 Kristiansen, T., 222
 Kristoffersen, J., 507, 512
 Kritchevsky, D., 395, 544
 Kritsky, G. A., 141
 Krivis, A., 261
 Krone, W., 169, 300, 303, 304, 580, 581, 582, 594
 Kronman, M. J., 473
 Krooth, R. S., 612, 615, 627, 628
 Kropf, A., 451
 Kröplin-Rueff, L., 199
 Krueger, A. P., 5
 Krueger, R. C., 257, 557
 Krueger, S., 18
 Kruglikova, K. E., 141
 Kruh, J., 296, 312, 317
 Krumm, D. L., 607
 Kruse, P. F., Jr., 606, 607, 608, 615, 627
 Kruse Jacobsen, B., 463
 Krzeminski, L. F., 541
 Ku, Y., 241
 Kubo, H., 34, 37

- Kubo, S., 484
 Kudo, T., 600
 Kueder, S., 261
 Kuenne, D. J., 52
 Kuhn, L. P., 57
 Kuhn, R., 84, 375
 Kuhn, W. L., 536
 Kukais, A., 513
 Kulaev, I. S., 141
 Kulikova, L. G., 141
 Kumar, A., 550
 Kumar, S. R., 79
 Kumar, V., 79
 Kumatori, T., 181, 631
 Kumin, S., 550
 Kummerow, F. A., 72, 79
 Kumta, U. S., 328, 332
 Kun, E., 243, 398
 Kunitz, M., 4, 9
 Kunkel, H. G., 549, 560, 561
 Kuno, S., 260
 Kupiecki, F. P., 526
 Kuppaswamy, S., 341
 Kurahashi, K., 231
 Kuretdni, K., 580
 Kurihara, K., 274, 563
 Kuriki, Y., 142
 Kurkov, V., 143
 Kurland, C. G., 309
 Kurokawa, M., 87
 Kuron, G. W., 537
 Kuru, M., 610
 Kurz, H., 563
 Kusai, K., 568
 Kutaček, M., 385
 Kutsky, R. J., 610
 Kuwabara, S., 610, 615, 623, 628
 Kuwada, S., 413, 414, 415, 418, 419, 420
 Kuyama, S., 413
 Kuzemakina, A. Z., 141
 Kuzin, A. M., 141
 Kuznetsova, N. V., 141
 Kwietny, H., 36
- L
- Labbe, R. F., 551, 555, 557
 Labby, D. H., 246
 Labouesse, J., 405
 La Brosse, E. H., 258
 Lack, L., 550
 Lacko, L., 218
 Lacks, S., 315, 641
 LaCour, L. F., 674
 Lacy, D., 663
 Ladd, J. N., 201, 438, 439
 Ladell, W. S. S., 336
 LaDu, B. N., 257, 387
 Laferte, R. O., 21
 Lagarrigue, M., 490
 Lagerkvist, U., 305
 La Grutta, G., 681
 Lagunoff, D., 624
 Lahay, E. M., 340
 Laidman, D. L., 452, 542
 Laird, C., 662
 Lajtha, L. G., 174, 181, 616, 631
 Laki, K., 475, 484, 485, 486, 491
 Laksesvela, B., 336
 Lal, S. B., 336
 Laland, S., 51
 Lambert, G. F., 330
 Lambertsen, G., 448
 Lamborg, M. R., 297
 Lamfrom, H., 297, 312, 313, 314
 Lamm, M., 456
 Lampen, J. O., 136, 217
 Lamprecht, W., 230
 Lamy, F., 610
 Landau, B. R., 218, 219, 233
 Landau, J. V., 684
 Landherr, C. A., 87
 Landowne, R. A., 72
 Lands, W. E. M., 199
 Landsteiner, K., 2
 Lane, D., 648
 Lane, M. D., 202, 203, 388
 Langan, T. A., 35
 Langdon, R. G., 189, 196, 200, 203, 206, 537
 Langemann, A., 463, 542, 553
 Langer, L., 180
 Langer, L. J., 499, 511, 516
 Langley, W. D., 490
 Langridge, R., 117, 119, 158, 647
 Lanman, G., 501, 507
 Lanman, J., 500
 Lanni, F., 651
 Lannon, T. J., 29
 Lanthier, A., 503, 505
 Larrabee, A. R., 255, 440
 Lardy, H. A., 50, 558
 Lareau, J., 233
 Lark, K. G., 671
 Larner, J., 223, 224, 225, 356, 362, 366, 370, 373
 Larralde, J., 220
 Larsen, H., 568
 Larson, B. L., 612, 623, 625
 Larson, E., 624
 Lascelles, J., 422, 550, 551
 Laser, H., 216
 Lasfargue, E. Y., 610
 Lash, J., 626, 630
 Laskowski, M., 154, 285, 286, 287
 Lasser, N. L., 24
 Lassio, A., 245
 Laszlo, J., 228
 Latarjet, R., 662
 Latta, M. J., 25
 Laufer, M. A., 184, 662
 Laughland, D. H., 455
 Laughlin, M. W., 536
 Laule, G., 358, 364
 Laurell, S., 204
 Laurence, D. J. R., 645
 Laurent, G., 560
 Laurent, T. C., 126
 Laver, W. G., 550
 Lavik, P. S., 631
 Lavine, L. R., 95
 Law, J., 528
 Law, J. H., 81, 83
 Law, L. W., 662
 Law, M. D., 207
 Lawler, H. C., 482
 Lawley, P. D., 138
 Lawson, D. E. M., 543
 Lazarus, S. S., 331
 Lea, C. H., 336, 402
 Leaback, D. H., 374
 Leach, S. J., 284, 285, 287
 Leadbeater, L., 476
 Leaf, G., 566
 Leahy, J., 300, 312
 Leanza, W. J., 433
 Leavan, A., 630
 Leavens, D., 75
 Lebedev, B. L., 45
 Lebedev, L. A., 101
 Leblond, C. P., 173
 Leboeuf, B., 192, 204, 205, 222
 Leclerc, M., 357
 Ledeen, R., 51
 Leder, I. G., 428, 429, 431
 Lederberg, J., 624
 Lederer, E., 71, 75, 76, 78, 82, 83, 85, 86, 87
 Ledingham, G. A., 72, 78
 Ledoux, L., 616
 Lee, J. B., 48, 51
 Lee, S. H., 403
 Lee, W. W., 134
 Lee, Y. C. P., 403, 490
 Lee, Y. P., 364, 491
 Leech, R. M., 568
 Leeds, W. E., 647
 Leeming, P. R., 76
 Leeson, J. A., 259
 LeFevre, P. G., 214, 218
 Leftin, J. H., 46
 Legallais, F., 610, 627, 629
 Le Goff, E., 553
 Lehman, F. E., 670
 Lehman, I. R., 21, 22, 141, 177, 178, 179
 Lehman, R. W., 447, 448
 Lehmann, H., 560, 561, 562
 Lehniger, A. L., 11, 23, 25, 26, 27, 28, 29, 34, 201, 594
 Leibenzon, A. S., 610
 Leibowitz, J., 65, 366
 Leidy, G., 629
 Leighton, J., 619, 627, 629
 Leikhim, E., 281
 Leikina, J. M., 141

- Leimgruber, W., 553
 Leloir, L. F., 142, 223,
 224, 359, 360, 362, 363,
 365, 366
 Lelouchier-Dagnellie, H.,
 247
 Lemack, N. I., 335
 Lemazhikhin, B. K., 101
 Lemberg, R., 15, 19, 563
 LeMen, J., 584
 Lemieux, R. U., 59
 Lemon, H. M., 505, 508
 Lempfrid, H., 375
 Lenestad, H., 677
 Lengyel, P., 165, 202
 Lennarz, W. J., 196
 Lennox, E. S., 619, 624
 Lenta, P., 28
 Lenz, R. W., 65
 Leob, H., 339
 Leone, E., 202
 Leong, J. L., 22
 Lepage, M., 82
 Leppänen, V., 221
 Lerique, J., 491
 Lerner, A. B., 261
 Lerner, B., 82
 Lerner, L. J., 536
 Lesko, S. A., 663
 Leslie, I., 608, 615, 619,
 629
 Lester, D., 388
 Lester, R. L., 19, 22
 Lett, J. T., 644, 662
 Letters, R., 82, 136, 145,
 151
 Lettré, H., 670, 682
 Leung, Y. C., 96
 Leuthardt, F., 409, 412,
 433
 Lev, M., 463
 Levellie, G. A., 330, 331,
 332
 Levenberg, B., 257
 Levin, D. H., 233
 Levin, E. Y., 257
 Levin, G., 36
 Levine, H. B., 87
 Levine, I., 649
 Levine, L., 281
 Levine, M. L., 351
 Levine, S., 622, 649
 Levine, W. G., 260
 Levinthal, C., 643, 644,
 646, 647, 650, 651, 653
 Levintov, L., 606, 608,
 611, 612, 613, 614, 622,
 628
 Levitt, M., 276, 561
 Levitz, M., 511, 513
 Levvy, G. A., 45, 373, 374
 Levy, H. M., 482
 Levy, H. R., 527, 528, 532
 Levy, J. F., 26, 594
 Levy, L. K., 513
 Levy, M., 285, 607, 608,
 609, 611, 612, 613, 614,
 615, 616, 621, 622, 629,
 630
 Lewak, S., 51
 Lewbart, M. L., 514
 Lewin, L. M., 82, 392
 Lewis, S. E., 23
 Lewis, W. R., 388
 Leybold, K., 509, 511
 Li, C. H., 506
 Li, L. F., 539
 Liao, S., 516
 Libenson, L., 175
 Libenson, R. J., 141
 Lichstein, H. C., 391
 Lichtenstein, J., 297, 308,
 316, 581, 582, 594, 617,
 642
 Lichenstein, N., 249
 Lieberman, A. H., 506
 Lieberman, I., 608, 609,
 610, 611, 612, 614, 616,
 618, 619, 625, 626, 628,
 629
 Lieberman, S., 500, 503
 Liebold, B., 275, 561
 Liepins, H., 609, 628
 Lifson, S., 280
 Lilga, K. T., 28
 Lin, S., 386
 Lind, J., 500
 Lindsay, E. M., 71
 Lindberg, B., 45, 49, 50,
 51, 55, 56
 Lindberg, O., 26
 Linderström-Lang, K.,
 270, 281
 Lindgren, F. T., 189
 Lindley, H., 102
 Lindner, A., 671
 Lindquist, B., 326
 Lindsey, J., 94
 Lindstedt, G., 253
 Lindstedt, S., 253
 Ling, N. S., 260
 Link, W. E., 73
 Linker, A., 373, 374, 623
 Links, J., 460, 542
 Linn, B. O., 526, 532, 542,
 543
 Linnane, A. W., 28
 Liozner, L. D., 141
 Lipkin, D., 138, 139, 141,
 142
 Lipkind, J. B., 516
 Lipmann, F., 299, 300,
 301, 302, 307, 311, 312,
 318, 362, 366, 514
 Lippman, V., 374
 Lipscomb, W. N., 125
 Lipsett, M. B., 205
 Lipsett, M. N., 26, 27,
 157, 401, 650
 Lipshitz, R., 304, 310
 Lipsky, S. R., 72
 Liptchina, L. P., 141
 Liguori, A. M., 115, 116,
 560
 Lisan, P., 536
 Lister, L. M., 508
 Litman, R., 660, 661
 Litman, R. M., 295
 Litt, M., 304, 310, 648,
 649
 Littauer, U. Z., 158, 303,
 304, 309, 310, 316
 Little, B., 510
 Little, H. N., 417, 418
 Littlefield, J. W., 606,
 617, 649
 Littleton, C. D., 125
 Liu, T. Y., 75
 Llubimova, M. N., 477,
 499
 Livingstone, G. E., 335
 Lloyd, A. G., 63, 374
 Lluch, M., 220
 Loach, P. A., 585, 586
 Lobbe, R. F., 25
 Lockart, R. Z., Jr., 606,
 607, 608, 621
 Locker, R. H., 475, 477,
 484, 485
 Lockhead, A. E., 386
 Lockwood, W. H., 550,
 551, 552, 553, 554, 557
 Loeb, J., 3
 Loeb, M., 538
 Loeb, M. R., 617
 Loeffler, G., 201
 Loening, U. E., 24
 Loewus, F. A., 384
 Lotfield, R. B., 293,
 298, 302, 308
 Lohmar, R. L., 78
 Loke, K. H., 513
 Loken, M. K., 182
 Lombardo, M. E., 500,
 504, 506
 Lomer, T. R., 114
 Lonberg-Holm, K. K., 233
 London, I. M., 273
 Long, S. K., 87
 Long, T. A., 327
 Longchamp, J. E., 508
 Longenecker, J. B., 335
 Longuet-Higgins, H. C.,
 258, 644
 Longwell, A., 672, 683
 Loomis, W. D., 527, 528
 Looney, W. B., 182, 183
 Loper, J. C., 656
 Lorah, C. L., 535
 Lorand, L., 487, 488
 Lorch, E., 195, 202, 388,
 389
 Lorch, I. J., 669
 Los, J. M., 46
 Lossow, W. J., 201
 Lotmar, R., 385
 Loughlin, M. E., 334
 Louwrier, K. P., 228
 Loux, H. M., 419
 Love, D. S., 481
 Love, R., 674

Love, W., 125
 Lovell, B. J., 60
 Lovell, F. M., 126
 Loveless, A., 295, 661
 Lovelock, J. E., 72
 Lovenberg, W., 259, 260
 Low, H., 26
 Lowe, G. L., 166
 Lowe, M. B., 556
 Lowenstein, J. M., 142, 483
 Lowey, S., 477, 478
 Lowry, O. H., 227
 Lowther, D. A., 623
 Lowy, J., 475, 485
 Lu, C., 95
 Luborsky, S., 158, 187, 304
 Lucas, J. M., 230
 Luchnik, N. V., 141
 Lucis, O. J., 506
 Luck, J. M., 663
 Ludwig, G., 411, 414, 420
 Luetscher, J. A., 506
 Luick, J. R., 230
 Lukens, F. D. W., 204
 Lukens, L. N., 258, 386
 Lukomaskaia, N. K., 373
 Lukomaskaia, T. S., 355
 Lunaas, T., 511
 Lunberg, G., 358
 Lund, A., 449, 455
 Lund, R. O., 611
 Lundegardh, H., 15
 Lundh, G., 326
 Lunt, M. R., 361, 374
 Luschat, U., 453
 Lushbough, C. H., 327
 Lutton, E. S., 198
 Luzzati, A., 275, 563
 Luzzati, V., 102, 115, 120
 Luzzatto, L., 223
 Lwowaki, W., 553
 Lynen, F., 195, 196, 199, 201, 202, 388, 389, 439, 526, 528, 529, 530, 531
 Lynn, W. S., Jr., 191, 203, 204, 205, 206, 501
 Lyster, R. L. J., 271, 559, 567
 Lytell, A. A., 331
 Lytle, R. I., 619

M

Mallée, O., 308, 671
 McAfee, J. W., 329
 McAllan, A., 374
 McAllister, R. M., 629
 MacArthur, D. M., 115
 McBee, R. H., 349
 McCallum, G. H., 95
 McCarthy, B. J., 308
 McCarthy, E. A., 515
 McCarthy, R. E., 605, 625
 McCarty, K. S., 612, 613, 614, 615
 McCay, P. B., 384, 461, 490
 McClellan, A. L., 57
 McComb, R. B., 219
 McCoy, E. E., 226
 McCoy, T. A., 607, 608, 611, 615, 627, 628, 629
 McCulloch, E. A., 608, 627, 631
 McCully, M. T., 402
 Macdacea, L., 477, 491
 MacDonald, D. L., 49, 51, 62
 McDonald, M. R., 673, 674
 McDonald, P. B., 254
 MacDonald, S. F., 15, 552, 553, 555
 McDonald, T. R. R., 125
 MacDougall, L. C., 341
 McDowell, R. H., 45
 McElroy, W. D., 240
 McElya, A. B., 183
 McEvoy, D., 411
 McEvoy, F. J., 133
 McFadden, M. L., 285
 McFall, E., 298, 606, 616, 622
 Macfarlane, M. G., 82, 83
 McGavin, S., 98, 99
 McGeachin, H. M., 125
 McGeachin, R. L., 226, 372
 McGhie, J. F., 79
 McGill, B. B., 644, 645, 647
 McGillivray, C. H., 126
 McGillivray, W. A., 449
 McGuire, J. S., 508, 509
 Machado, R. D., 672, 680, 684
 McHale, D., 458, 541
 Machell, G., 54, 55
 Machlin, L. J., 462
 McInnes, A. G., 59, 73
 McIntire, F. C., 580
 McIntyre, J., 675
 McIsaac, W. M., 261
 MacKay, M., 94
 McKay, S. R., 338
 McKenna, J. M., 623, 624
 McKenzie, B. F., 395
 Mackenzie, C. G., 217
 Mackenzie, C. H., 459
 McKenzie, H. A., 286, 473, 477
 Mackenzie, J. B., 459
 MacKenzie, R. D., 536
 McKillican, M. E., 49
 Mackinney, G., 541
 Mackler, B., 11, 12, 25
 McLain, L. W., Jr., 195
 McLaren, H. D., 244
 McLaren, J. A., 411
 McLaughlin, J. M., 334
 McLean, J., 77
 MacLennan, A. P., 85, 87
 Macleod, H. L., 655
 MacLeod, R. M., 203, 204, 205, 206
 McLimans, W. F., 609
 McLoughlin, B. J., 544
 McLoughlin, V. C. R., 66
 McManus, E., 433
 McManus, F., 249
 McManus, I. R., 462, 490
 McMorris, C., 500, 506
 McMurray, W. C., 29
 McNally, S., 61
 McNelis, E., 433, 435
 McNutt, W. S., 366, 411, 418, 419
 McPhee, J. R., 558
 McPherson, H. T., 508
 MacPherson, I. A., 627
 McPherson, J. C., 190
 MacQuillan, A. M., 234
 McQuillen, K., 293, 308, 309
 McQuilkin, W. T., 607, 608, 610, 628
 MacRae, T. P., 101
 Macritchie, F., 58
 McSweeney, G. P., 543
 McWain, P., 133
 Madden, R. E., 333
 Maehly, A. C., 272
 Magasanik, B., 253, 308, 606, 616, 622
 Magdoff, B. E., 102, 124
 Magee, W. E., 614
 Magee, W. L., 75
 Mager, J., 240, 591, 592, 593, 597, 600
 Magnell, I., 433
 Magrath, D. I., 138
 Maguire, M. H., 137
 Mahadevan, V., 81
 Maher, J., 354
 Mahesh, V. B., 510, 511
 Mahler, H. R., 168, 170, 297, 318, 594, 658
 Mailman, E., 99
 Main, R. K., 649
 Maio, J. J., 220, 223, 358, 612, 622
 Majhofer-Oréscanin, B., 83
 Majumdar, B. N., 453
 Makinose, M., 487, 489
 Makita, M., 87
 Makus, Z., 71
 Malanga, C., 433
 Malcolm, B. R., 98
 Maley, F., 180
 Maley, G. F., 180, 413, 414, 415, 416, 418, 420
 Malhotra, O. P., 358, 370
 Malik, D. A., 337
 Malkin, T., 81
 Malmgren, H., 680
 Malmstrom, B. G., 36
 Mamalis, P., 458
 Mameesh, M. S., 465
 Manchester, K. L., 242, 249

- Manchon, P., 37
 Mancini, G., 580, 597, 598
 Mandel, H. G., 314
 Mandel, P., 24, 73, 87
 Mandelkern, L., 645
 Mandell, J. D., 646
 Mandelstam, J., 620
 Mandelstam, P., 226
 Mangiantini, M. T., 461
 Mangum, J. H., 568
 Mann, I., 336
 Mann, J. D., 258
 Mann, P. J. G., 245, 587
 Mann, P. F. E., 365
 Mann, S., 24
 Mann, T., 82, 229, 230
 Manners, D. J., 351, 355, 356
 Mano, Y., 248, 433
 Manson, L. A., 176, 608, 612, 613
 Mansour, T. E., 225, 260
 Mansour, T. W., 226
 Mantieva, V. L., 141
 Manwell, C., 558, 559, 560
 Manzo, E. Y., 75
 Mapson, L. W., 383
 Marano, B., 647
 Marcaud-Raeber, L., 486
 Marcia, J. A., 190
 Marcinkiewicz, S., 458
 Marcus, F., 490
 Marcus, P. I., 627
 Marcus, S. J., 75
 Marder, H. L., 48
 Marei, A. A., 49
 Margoliash, E., 16, 33, 34, 149, 564, 565, 566
 Margolis, S., 204, 205, 206
 Marinetti, G. V., 22, 73, 83, 199, 459, 564
 Marini, F., 217
 Marion, L., 584
 Marita, T., 299
 Markelova, I. V., 141
 Markert, C. L., 228, 560
 Markham, E., 553
 Markham, R., 119, 138, 139, 140, 141, 147, 306, 318
 Märki, F., 23, 464
 Markley, K., 527
 Markovitz, A., 363
 Marks, G. S., 15, 554, 555
 Marks, P. A., 34, 517
 Marmur, J., 158, 296, 316, 645, 646, 647, 648
 Marnati, M. P., 437
 Marquardt, H., 600
 Marré, E., 385
 Marrian, G. F., 513
 Marsch, C. A., 360
 Marsh, B. B., 489
 Marsh, C. A., 45, 374
 Marsh, J. B., 587
 Marsh, J. C., 177
 Marsh, J. M., 357
 Marsh, R. E., 96, 105
 Marshak, A., 171
 Marshall, B. A., 51
 Marshall, J. K., 214, 218
 Marshall, J. M., 474, 478
 Marshall, M., 247
 Marshaw, J. B., 195
 Marsico, J. W., 143
 Marsland, D., 669, 684
 Marston, R. Q., 624
 Martell, A. E., 242
 Martin, C. J., 383
 Martin, D. B., 193
 Martin, G. M., 260
 Martin, G. R., 384
 Martin, H. L., 480, 482, 487
 Martin, M., 11
 Martin, N., 111, 561
 Martin, W. H., Jr., 591
 Martinez, R. J., 374
 Martius, C., 23, 460, 464, 465, 542
 Martonosi, A., 475, 476, 478, 487
 Maruo, B., 372
 Maruyama, K., 477, 479
 Maruyama, Y., 671
 Marvin, D. A., 117, 647
 Marvin, J. F., 182
 Mascitelli-Coriandoli, E., 396, 404
 Maslen, E. N., 115
 Mason, H. S., 32, 257, 258, 259
 Mason, M., 260, 516
 Masri, M. S., 256, 257
 Massart, L., 592
 Massey, V., 12, 19, 20, 36, 245
 Massey-Westropp, R. A., 543
 Massieu, G. H., 336
 Massonet, R., 451
 Masuda, T., 413, 414, 415, 416, 418, 419, 420
 Matheson, A. T., 646, 662
 Mathias, A. P., 617
 Mathieson, A. McL., 95
 Mathur, S. C., 126
 Matschiner, J. T., 501
 Matsubara, H., 17
 Matsuda, G., 561, 562
 Matsukawa, T., 433
 Matsumiya, H., 479, 480, 482
 Matsumoto, J. J., 473, 479, 480
 Matsuoaka, Y., 413
 Matsushima, T., 355
 Mattern, C. F. T., 438, 439
 Matterson, L. D., 22
 Matthes, K. J., 206
 Matthews, R. E. F., 295, 643
 Mattison, F. H., 198
 Matuda, K., 610
 Matzelt, D., 612
 Maudgal, R. K., 533
 Mauritzen, C. M., 663
 Mauzerall, D., 550, 551, 553, 554, 555, 556, 557
 Maxwell, E., 515
 Maxwell, E. S., 231, 359
 Maxwell, M., 607, 608, 627, 628, 629
 May, C. D., 326, 336
 Mayer, F. C., 366, 370
 Mayer, R. M., 207
 Mazen-Knobloch, A., 120
 Mazia, D., 296, 669, 670, 671, 672, 673, 675, 676, 677, 678, 680, 681, 683
 Mazunder, R., 202
 Mazur, A., 385
 Mazzacca, G., 612
 Mazzioti di Celso, P., 448
 Meade, C. H. J., 412
 Meadow, P., 252
 Meakins, G. D., 77, 78
 Mechanic, G., 254
 Medina, A., 240
 Medina, V. J., 36
 Mehlretter, C., 46, 50
 Mehta, R., 169, 439
 Meijering, C. A., 560
 Meilman, E., 483
 Meisel, N. M., 141
 Meisky, K. H., 462
 Meister, A., 37, 245, 249, 300, 302, 490
 Melampy, R. M., 503
 Melechen, N. E., 316
 Melnick, I., 557
 Melvin, E. H., 74, 75, 78
 Mendelssohn, J., 308
 Mendes, C. B., 340
 Menges, R., 366
 Menne, F., 248
 Menon, G. K. K., 200, 201, 202, 204
 Mercer, E. H., 101
 Mercer, E. I., 543
 Merchant, D. J., 606, 615, 624
 Mészáros, O., 45, 51
 Mering, J., 125
 Merrill, S. H., 140, 307
 Merritt, F. L., 204
 Merselis, J. E., 465
 Mertz, E. T., 327, 329, 334
 Mertz, W., 21, 460
 Merwin, R. M., 627, 630
 Merz, H., 144
 Meselson, M., 315, 316, 646
 Meshkova, N. P., 490
 Metcalf, W., 242
 Metta, V. C., 330, 335
 Metznerberg, R. L., 247
 Metzgar, D. P., Jr., 610, 628
 Metzler, D. E., 433
 Meyer, A. I., 245

- Meyer, J., 457
 Meyer, K., 373, 374, 623
 Meyer, K. H., 126
 Meystre, C., 506
 Michael, P. J., 502
 Michalec, C., 71
 Michel, G., 75, 78, 86
 Michelson, A. M., 133, 136, 139, 140, 143, 144, 145, 146, 147, 149, 150, 151, 154, 155, 156, 157
 Micou, J., 172, 173, 617
 Middlebrook, W. R., 478
 Middleton, E. J., 334
 Migicovsky, B. B., 457
 Migita, M., 473, 479
 Migliacci, A., 437
 Mihalyi, E., 478, 491
 Mihara, S., 681, 682
 Mihich, E., 404
 Mil, S., 166
 Mikadze, G. V., 479
 Milch, R. A., 259
 Milhorat, A. T., 460
 Miller, A. K., 592
 Miller, C. S., 434
 Miller, D., 219, 220
 Miller, D. S., 339
 Miller, I. M., 435
 Miller, K. D., 355
 Miller, L. F., 619
 Miller, L. L., 473, 620
 Miller, O. N., 73, 396, 540
 Miller, W. G., 247
 Millin, D. J., 79
 Millionova, M. I., 99
 Millman, B. M., 485
 Millmann, N., 451
 Mills, G. C., 30
 Mills, G. T., 363
 Mills, J. A., 62
 Mills, J. F. D., 126
 Mills, S. E., 612, 626
 Milman, L. S., 141
 Mitlofsky, L., 618
 Milstein, J., 192
 Minakami, S., 565, 566, 557
 Minghetti, A., 177, 355, 437
 Mingioli, E. S., 250, 440
 Minnaert, K., 13, 27
 Minnick, N., 326
 Minoura, J., 411
 Minthorn, M. L., Jr., 251
 Mirovich, N. I., 477, 479, 482, 486
 Mirsky, A. E., 297, 308
 Misch, L., 126
 Mistry, S. P., 201, 248, 390, 391, 439
 Mitamura, K., 610, 611, 614, 627, 628
 Mitchell, D. L., 49
 Mitchell, E. R., 477
 Mitchell, H. H., 334
 Mitchell, H. K., 412, 421
 Mitchell, J. E., 513
 Mitchell, K. G., 394
 Mitchell, P., 214
 Mitchison, J. M., 672, 679, 684
 Mitoma, C., 254
 Mitra, A. K., 47
 Mix, K., 48
 Miyakawa, T., 71
 Miyaki, K., 597, 600
 Miyazaki, E., 479, 482
 Mizuhara, S., 433, 434
 Mizushima, H., 17, 566
 Moat, A. G., 165, 390
 Mochida, T., 600
 Moe, J. G., 461
 Moeda, A., 309
 Moffatt, J. G., 143
 Moggian, G., 598
 Mogridge, R. C. G., 431
 Mohr, E., 364, 375
 Moiseeva, V. P., 486
 Mokrasch, I. C., 248
 Moldave, K., 300, 302, 311
 Mole-Bajer, J., 674, 682
 Müller, E. F., 421
 Müller, F., 228, 560
 Molnar, J., 487, 488
 Mommaerts, W. F. H. M., 224, 362, 475, 477, 487
 Moncloa, F., 502
 Mondovi, B., 242
 Monier, R., 184, 303, 304, 305, 307, 310
 Monod, J., 214, 215, 216, 234, 297, 298, 314, 315, 316, 351, 357, 358, 655
 Monson, W. J., 337
 Montag, B. J., 617
 Montano, E. L., 341
 Montgomery, P. O., 663
 Monty, K. J., 201, 439
 Moody, G. O., 65
 Moon, H. D., 611, 619
 Moore, A. E., 133, 612, 627, 630
 Moore, C. G., 543
 Moore, C. V., 557
 Moore, E. C., 175
 Moore, F. D., 511
 Moore, K. B., 460
 Moore, R. H., 46
 Moore, S., 102, 269, 277, 278, 558
 Moore, T., 460, 462
 Moorehead, P., 631
 Moorehead, P. S., 627, 630
 Moos, C., 461, 487, 488
 Morales, M. F., 477, 479, 482
 Moran, T., 337
 Morell, D. B., 563
 Morgan, H. E., 218, 221
 Morgan, J. F., 234, 605, 607, 608, 609, 612, 613, 615
 Morgan, R. S., 118, 119
 Morgantini, F., 580, 598
 Morgen, S., 33
 Mori, K., 45
 Mori, T., 568
 Morice, I. M., 75
 Morikawa, I., 564, 568
 Morimura, Y., 681, 682
 Morino, Y., 402
 Morisue, T., 402
 Morita, F., 479, 480, 482
 Moritz, U., 623
 Moroson, H., 662
 Morosova, T. M., 141
 Morrill, G. A., 277, 278
 Morris, A., 66
 Morris, L. J., 78
 Morris, M., 241
 Morris, N. R., 175
 Morris, R. A., 73
 Morris, W. W., 448, 454
 Morrison, A. B., 334, 335
 Morrison, J. D., 126
 Morrison, J. F., 489
 Morrison, M., 11, 15, 19, 20, 22, 23, 27, 28, 459, 549, 560, 564, 565
 Morrison, M. A., 332
 Morrison, P. A., 158
 Morrison, R. A., 167, 304
 Morsingh, F., 553
 Mortenson, L. F., 239
 Mortimer, P. I., 253
 Morton, D. J., 248
 Morton, H. J., 234, 607, 609, 612, 613
 Morton, R. A., 449, 452, 460, 542, 543
 Morton, R. K., 17, 19, 228, 231, 549, 568
 Moscatelli, E. A., 73
 Moscona, A., 626
 Moses, M. J., 673
 Mosettig, E., 536
 Mosevitsky, M. I., 158
 Moskowitz, M., 610, 628
 Moslein, E. M., 528, 530
 Mosolov, A. N., 141
 Moss, B., 143
 Mossel, D. A. A., 220
 Mosser, D. G., 182
 Mota, M., 672, 673, 682
 Mott, J. C., 597
 Mountain, J. T., 539
 Mourikides, G. A., 251
 Mower, H. F., 239
 Moye, C. J., 415
 Moyed, H. S., 247, 253
 Moyer, J. D., 64
 Mudd, J. B., 32
 Mudd, S. H., 255, 586
 Mueller, G. C., 516, 611, 615
 Mueller, H., 478, 480, 488
 Muhrad, A., 476
 Muirhead, H., 102, 111, 113, 269, 271, 559, 560
 Mukherjee, K. L., 339
 Mulcare, D., 329

Muldrey, J. E., 73
 Muller, C. J., 277, 561
 Muller, R., III, 275, 561
 Mulman, L. S., 141
 Mulrow, P. J., 500, 504, 506
 Mumma, R., 82
 Munch, T., 448
 Munch-Petersen, A., 438, 439
 Munch, A. U., 505, 517
 Munday, J. S., 244, 517
 Mundry, K. W., 7, 299, 661
 Munier, R., 302
 Munkner, C., 205
 Munro, H. N., 327
 Munstermann, A.-M., 507
 Munyon, W. H., 615
 Murakami, W. T., 649
 Muraki, K., 581, 598
 Murayama, C., 275
 Murayama, M., 271, 558, 562
 Murphy, W. H., 606
 Murray, D. H., 51
 Murray, J., 490, 491
 Murray, K. E., 73, 79
 Murray, M. R., 610
 Murray, R., 564
 Murray, R. G. E., 669
 Murty, N. L., 72
 Muscatello, U., 486
 Mycek, M. J., 584, 590
 Myers, D. K., 182
 Myers, L. S., 272
 Myers, S. M., 333
 Myers, T. C., 481
 Myklestad, H., 448
 Myrbäck, K., II, 373, 433, 549
 Myrvik, Q. N., 632

N

Nabors, C., Jr., 503, 505
 510, 511, 512
 Nabors, C. S., 618
 Nachlas, M. M., 24
 Nadeau, G., 258
 Nadel, E. M., 512
 Nadkarni, G. B., 251
 Naegle, S., 192
 Nagabhushanan, A., 355
 Nagai, T., 479, 482, 487, 489
 Nagasawa, K., 51
 Nagatsu, T., 37, 258
 Nagatsum, I., 34
 Nagy, E., 476
 Nahinsky, P., 556
 Naismith, J. D., 327
 Najjar, V. A., 226
 Nakada, I. H., 374
 Nakagawa, I., 329
 Nakagawa, S., 581, 598
 Nakahara, A., 559
 Nakai, M., 568
 Nakajima, T., 675
 Nakamoto, K., 242
 Nakamura, M., 45, 256
 Nakamura, S., 536
 Nakamura, T., 13
 Nakamura, Y., 355
 Nakayama, H., 429, 430, 431, 432
 Nakazawa, Y., 83
 Nakomoto, T., 35, 243
 Nall, D. M., 511
 Nandi, J., 506
 Nankina, V. P., 658
 Nanninga, L. B., 480, 483
 Naono, S., 314, 315, 316
 Naora, H., 172
 Nardelli, M., 97
 Nasatir, M., 672, 680
 Nason, A., 21, 22, 240, 459, 460
 Nass, S., 672
 Nasset, E. S., 333
 Nasu, K., 597
 Nasuti, F., 390
 Nathans, D., 241, 311
 Nawa, S., 412
 Nebel, B. R., 673
 Needham, D. M., 473, 474, 477, 479, 481, 486, 491
 Neel, J. V., 560
 Neely, W. B., 45, 46, 349, 350, 371
 Neeman, M., 64
 Negishi, T., 83
 Neher, R., 501, 503, 504, 506, 507, 510, 512, 513, 517
 Nehorayan, S., 461
 Neiderhiser, D. H., 535
 Neidhardt, F. C., 308
 Neidle, A., 584, 590
 Neilands, J. B., 252, 557, 564, 567, 568
 Neilsen, H., 433
 Neimann-Sørensen, A., 449, 453, 454
 Neimark, J. M., 609, 611
 Nekhorocheff, J., 491
 Nelson, G. J., 74
 Nelson, N. M., 349
 Nelson, T. S., 466
 Nemer, M. J., 250, 251
 Nemeth, A. M., 24, 30, 246, 329, 397
 Nesemann, G., 512
 Nesheim, M. C., 461
 Ness, R. K., 51, 133
 Neuberger, A., 252, 284, 332, 549, 550, 551
 Neufeld, E. F., 231, 361, 362, 681
 Neufeldt, I., 201
 Neuhaus, F. C., 251
 Neuman, R. E., 331, 607, 608, 609, 610, 611, 612, 613, 614, 627, 629
 Neuman, M. W., 387, 456
 Neumann, N. P., 568
 Neve, R. A., 551, 555
 Newey, H., 220
 Newhaus, F. C., 302
 Newmeyer, D., 427
 Newton, A. A., 491, 617, 673
 Newton, J. W., 568
 Nguyen-Chi, J. P., 400
 Nguyen-Philippon, C., 400
 Nguyen-Trung-Luong Cros, H., 86
 Nichol, C. A., 404, 515
 Nicholas, D. J. D., 240
 Nichols, A. V., 189
 Nichols, T., 74
 Nicholson, I. W., 355
 Nickel, W. F., Jr., 333
 Nickell, E. C., 74
 Nickon, A., 21, 22
 Nicol, B. M., 327
 Nicol, M., 452
 Nicolajeff, A., 120
 Nicolson, A., 50
 Nielsen, G. K., 208
 Nielsen, K., 82
 Nieradt-Hiebsch, C., 34
 Nigam, V. N., 355
 Nigrelli, F., 51
 Nikiforova, G. S., 141
 Nilsson, K., 587
 Nilsson, W. A., 74
 Nimmo-Smith, R. H., 422
 Nimz, H., 51
 Nirenberg, M. W., 219, 226, 228
 Nishida, G., 557
 Nishimura, M., 450, 568
 Nisman, B., 302, 318
 Nisselbaum, J. S., 34, 228, 595, 600
 Nissim, J. A., 220
 Nitz-Litzow, D., 465
 Niu, C. I., 485, 486
 Nixizawa, K., 357
 Nixon, D. A., 221
 Nixon, H. L., 122, 124
 Njaa, L. R., 448
 Noall, M. W., 242
 Nobel, S., 456
 Nobless, C., 352
 Nocke, L., 513
 Noda, H., 420, 423, 424, 479
 Noelken, M. E., 479
 Nogueira, O. C., 16
 Nohara, H., 299
 Nojima, S., 82
 Nolan, H., 516
 Nolan, M. F., 221
 Noltmann, E., 227
 Nomizu, H., 71
 Nomura, M., 316
 Nomura, T., 76
 Norberg, E., 351
 Nordin, P., 351
 Nordman, C. E., 125

- Norgard, D. W., 541
 Norman, A., 662
 Norris, C. B., 673
 Norris, L. C., 466
 North, A. C. T., 98, 99,
 102, 111, 112, 113, 114, 269,
 271, 559, 560
 Northrop, J. H., 1, 3, 4, 5,
 6, 7, 9
 Norton, H. W., 330
 Norton, J. S., 528, 538
 Norton, P. M., 326, 329,
 339
 Nose, Y., 428, 429, 431,
 514
 Nossal, G. J. V., 624
 Novelli, G. D., 293, 297,
 300, 302, 435
 Novick, A., 216
 Novogradsky, S., 33, 34
 Nowakowska, J., 74
 Nowell, P. C., 624
 Noyes, W. D., 181, 616
 Nozaki, M., 17, 564, 565, 566
 Nubel, G., 413
 Nuenke, B. J., 435
 Numata, K., 454
 Nunn, R. F., 207
 Nunnikoven, R., 566
 Nurnberger, H., 34
 Nyberg, S. C., 126
 Nygaard, A. P., 18, 34, 228
 Nyholm, B., 510
 Nyman, M. A., 505, 507

 O
 Oaks, W., 536
 Oates, J. A., 259
 O'Barr, T. P., 241
 Oberling, C., 630
 O'Brien, J. R. P., 273, 563
 Ochl, H., 454
 Ochoa, S., 119, 165, 167,
 201, 202, 317
 O'Connell, E. L., 227
 O'Connor, R., 245
 Odagiri, S., 453
 O'Dell, B. L., 393
 Odell, W. D., 204, 205
 Odinzova, M. S., 141
 Oertel, G., 506, 507
 Oestergren, G., 682
 Oesterling, M. J., 330
 Ofengand, E. J., 169, 299,
 300, 301, 303, 304, 305,
 307, 310
 Officer, J. E., 631
 Ogata, A., 583
 Ogata, K., 299
 Ogawa, S., 454
 Ogryzolo, M. A., 205
 Ogura, Y., 18, 19, 568
 O'Hagan, J. E., 271, 272,
 559, 563
 Ohnishi, E., 250
 Okada, H., 217
 Okada, M., 503, 513
 Okada, S., 183
 Okada, T. S., 626
 O'Kane, D. J., 435, 441
 Okaya, Y., 126
 Okazaki, R., 142, 363
 Okey, R., 195
 Okuda, K., 438
 Okuda, Y., 396
 Okumura, H., 627, 630
 Okun, L., 662
 Okunuki, K., 12, 13, 17, 19,
 20, 564, 566, 568
 Olavarria, J. M., 223, 362,
 363
 Olcott, I., 455
 O'Leary, W. M., 75
 Oleazalel, T., 142
 Oliver, R., 174, 181, 616,
 631
 Olmsted, P. S., 75, 166
 Olsen, A. G., 507
 Olsen, E. M., 449
 Olson, R. E., 195, 477,
 542, 543
 O'Meara, D., 55
 O'Neal, R. M., 189
 Onions, W. J., 101
 Ono, K., 189
 Ono, S., 398
 Ono, T., 615
 Ooi, T., 475, 476
 Oosawa, F., 475, 476
 Opara-Kupinska, Z., 660
 Oparin, A. I., 141
 Oppel, V. V., 476
 Ord, M. G., 181, 182
 Orgel, L. E., 549, 651, 658
 Orii, Y., 19, 20
 Oroshnik, W., 451
 Orr, H. C., 619, 627, 629
 Orth, R. D., 204, 205
 Ortiz, J. M., 402
 Ortiz, P. J., 119
 Ory, R. L., 72
 Osawa, S., 297, 303, 304,
 305, 308, 310, 663
 Osbahr, A. J., 462
 Osbond, J. M., 79
 Osborn, M. J., 425, 426,
 441
 Oshima, Y., 217
 Osinski, P. A., 510
 Ostashever, A. S., 402
 Otarova, G. K., 482
 Oth, A., 647
 Otsuka, H., 682
 Otsuki, I., 610, 611, 614
 Ott, W. H., 433, 537, 553,
 554
 Ottesen, M., 287
 Otto, G. F., 466
 Ottonienghi, O., 217
 Ottoson, R., 680
 Oughton, B. M., 95, 97
 Oura, E., 433
 Ousterhout, L. E., 331
 Ove, P., 608, 609, 610, 611,
 612, 614, 616, 618, 619,
 625, 626, 628, 629
 Ovenfor, C., 337
 Overath, P., 201, 388, 439,
 526
 Overbeek, J. T. G., 301
 Overend, W. G., 45, 50, 51,
 57, 60, 63, 64
 Owen, L. N., 51
 Oyama, V. I., 606, 607,
 608, 611, 612, 613, 616,
 620, 621, 622, 629
 Ozawa, T., 37, 165, 245
 Ozeki, H., 656, 658
 Ozzello, L., 610

 P
 Pacák, J., 62
 Pace, J., 337
 Pacha, W. L., 245
 Packer, L., 26, 27, 28,
 215, 218, 615, 616
 Padilla, A. M., 339
 Padilla, G. M., 682
 Page, A. C., Jr., 22, 543
 Page, I. H., 261
 Pahl, H. B., 140, 182
 Paine, C. M., 241
 Painter, R. B., 172, 174,
 617, 621, 631, 680
 Painter, T. J., 63
 Pakhomova, M. V., 141
 Palade, G. E., 309, 475,
 488
 Palay, S. L., 189, 191
 Paléus, S., 564, 566, 567,
 568
 Palleroni, N. J., 348
 Pallila, F., 284
 Palludan, B., 449, 453,
 454
 Palmer, G., 245
 Palopoli, F. P., 536
 Pamijans, V., 249
 Pan, C. H., 485
 Pan, S. C., 355
 Pandolfo, L., 457
 Pangborn, M. C., 82
 Pantulu, A. J., 337
 Papa, M. J., 615, 616
 Pappas, G. D., 623
 Pappenheimer, A. M., Jr.,
 374, 619
 Pardee, A. B., 214, 234,
 293, 295, 297, 298, 302,
 314, 317, 654, 655, 660
 Pare, C. M. B., 261
 Parihar, D. B., 136
 Park, C. R., 218, 221
 Park, E. A., 456, 457
 Park, R. B., 228, 543
 Parker, C. A., 239
 Parker, C. J., 489, 490
 Parker, F. S., 46
 Parker, R. C., 608, 620,

- 627, 631
Parker, R. F., 607, 608, 609, 615, 625
Parker, V. H., 29
Parkes, A. S., 403
Parkinson, L. R., 335
Parks, J. M., 277
Parks, J. T., 142
Parks, R. E., Jr., 246, 625
Parmar, S. S., 24
Parnell, J. P., 449
Parr, C. W., 226
Parr, L. J., 336
Parrish, D. B., 448
Parrish, R. G., 105, 270
Parrot, J., 387
Parr, G. S., 114, 125
Parshina, E. A., 479, 482, 486
Parsons, B. J., 220
Parsons, D. S., 220
Parsons, W. B., Jr., 396, 540
Partridge, C. W. H., 653
Pasher, I., 440
Pasieka, A. E., 607, 608, 612, 613
Passinsky, A. G., 141
Passonneau, J. V., 227
Pätau, K., 671
Paterson, J. Y. F., 452, 542
Patragnani, N., 16
Patrick, A. D., 351
Patrick, S. J., 339
Patterson, A. L., 125
Patterson, M. K., Jr., 435
Patwardhan, M. V., 243, 337, 338, 398
Paul, J., 606, 615, 616, 619, 620, 621, 626, 631
Paul, K.-G., 33, 563, 564, 567, 568
Paul, M. B., 644
Paul, W., 387
Pauling, L., 96, 97, 100, 101, 108, 114, 271, 275, 560, 561
Paulsen, H., 50
Paulus, H., 196, 197, 198, 392
Paulus, W., 411, 414, 420
Pautard, F. G. E., 101
Pav, J., 191, 205
Pavlovskis, O. R., 622
Pawan, G. L. S., 204
Pawelkiewicz, J., 550
Payner, A. H., 183
Paynter, O. E., 337
Pazur, J. H., 351, 354, 355, 357, 363
Peacock, N., 101
Peacocke, A. R., 155, 662
Pearson, C. M., 222, 224, 230, 362
Pearson, E. S., 615
Pearson, W. N., 325, 387
Pease, D. C., 677
Peat, S., 351, 372
Peaud-Lenoël, C., 352, 352, 353, 359, 361, 365, 368, 371
Pechet, M. M., 510
Pedersen, C., 51
Pelc, S. R., 181, 453
Pelczar, M. J., Jr., 591
Pelevina, I. I., 141
Pelling, G., 174
Pelter, A., 534
Pendlington, S., 411, 412, 418, 419
Penfold, B. R., 95
Pen'kina, V. S., 101
Penn, N., 12
Penn, N. W., 169
Pennell, R. B., 609
Pennock, J. F., 452, 542
Penttinen, K., 619, 627
Pepé, F., 474, 478
Pepinsky, R., 95, 115, 126
Peppers, E. V., 606, 607, 611, 612, 614, 615, 616, 617, 624
Peraino, C., 333
Perdue, H. S., 464, 466
Perevoshchikova, K. A., 141
Perez-Milan, H., 256
Perkins, D. D., 427
Perkins, H. R., 374
Perles, R., 394
Perlin, A. S., 47, 56, 62
Perlmán, D., 618, 619, 625
Perlmann, G. E., 286, 563
Perlmutter-Hayman, B., 50
Peron, F. G., 138, 502
Perrin, D. D., 271
Perrin, J., 73
Perron, R., 71
Perry, M. D., 215, 218
Perry, R. B., 617
Perry, R. P., 27, 173
Perry, S. V., 473, 474, 476, 477, 478, 479, 480, 483, 484, 485, 486, 488, 489, 491
Perry, W. F., 395, 396, 540
Perryman, N. C., 191
Persidsky, M. D., 682
Perske, W. F., 625
Persky, A., 50
Persky, S., 587, 588, 589
Perutz, M. F., 93, 94, 96, 97, 102, 103, 104, 105, 111, 112, 113, 114, 118, 119, 122, 269, 271, 276, 559, 560
Pesch, L., 515
Pessolano, A. A., 433
Petek, F., 349, 359
Peter, P. A., 620
Peters, J. M., 426
Peters, L., 592
Peters, R. A., 75
Petersen, G. B., 642
Peterson, E. A., 140, 276
Peterson, E. W., 259, 336
Peterson, G. B., 295
Peterson, J., 95, 97
Peterson, R. E., 508, 510
Peterson, W. E., 360
Petit, J. F., 28
Petitpas, T., 125
Petix, J., 15, 19
Petrasek, R., 234
Pfeiffer, H. H., 676
Pieningsdorf, G., 455, 456
Piffner, J. J., 437
Pfleiderer, G., 34, 144, 413
Phaff, H. J., 373
Phansalkar, S. V., 326, 337
Phillips, A. W., 364
Phillips, A. H., 189, 528
Phillips, D. C., 102, 105, 106, 108, 109, 270, 559, 560
Phillips, D. P. M., 648, 649, 663
Phillips, E. J., 339
Phillips, G. O., 65
Phillips, H. J., 615, 629
Phillips, J. G., 501, 506
Phillips, J. N., 271, 556
Phillips, P. G., 336
Phillips, T. L., 624
Phillips, W. E. J., 455, 460
Philpott, D. E., 101, 474, 475, 478, 672
Piatelli, M., 15
Piatnek, D., 477
Pickering, R. A., 58
Pickworth, J., 94, 126
Pierard, A., 244
Pierce, D. A., 241
Piette, L., 32
Piez, K. A., 606, 607, 608, 611, 612, 613, 614, 620, 621, 622, 628
Pike, R. L., 403
Pileggi, V. J., 502
Pileri, A., 616
Pilsun, J. F. V., 489, 490
Pimentel, G. C., 57
Pinchot, G. B., 28
Pincus, G., 502
Pineda, O., 341
Pinkard, R. M., 46, 49
Pinkernelle, W., 584
Pirie, A., 451
Pirie, N. W., 337
Pirrie, R., 566
Pittard, A. J., 247
Pittet, A. O., 47
Pixley, B. Q., 404, 550
Pizer, L. I., 176, 228, 392
Plack, P. A., 448

- Plagemann, P. G. W., 34, 228
 Planel, H., 619, 624
 Planterose, D. N., 23
 Platt, F. W., 305
 Plaut, G. W. E., 409, 410, 411, 413, 414, 415, 416, 417, 418, 419, 420, 422
 Plaut, W., 172, 174, 297
 Plesner, P., 679
 Plessing, H., 454
 Pocchiari, F., 233
 Poey Seng Hin, 338
 Poglazov, B. F., 479
 Pohlen, E. K., 52
 Pohowalla, J. N., 341
 Porkorný, J., 87
 Pol, E. H., 119, 140, 147
 Polgar, N., 79
 Pollack, M. E., 631
 Pollard, C. J., 21, 22, 448, 450, 459, 460, 463
 Pomeranz, S., 257
 Pomoschnikova, N. A., 141
 Pon, N. G., 228
 Ponsold, W., 455
 Pontecorvo, G., 657
 Pontis, H. G., 142, 363
 Pontremoli, S., 226, 232
 Ponz, F., 220
 Popjak, G., 527, 528, 530, 531, 532, 534, 541, 544
 Porcellati, G., 73
 Porra, R. J., 549, 555
 Porter, J. W., 193, 525, 526, 527, 541
 Porter, K. R., 488, 623, 671, 672, 675, 680, 684
 Portius, N. J., 511
 Porto, F., 32
 Portzehl, H., 477, 487, 488
 Post, B., 96
 Post, R. L., 221
 Posternak, T., 50, 51, 393
 Postgate, J. R., 568
 Potier, P., 584
 Potter, B. A., 225
 Potter, R. L., 142, 172, 183
 Potter, V. R., 180
 Pourchez, A., 71
 Pour-El, A., 156
 Poverenny, A. M., 141
 Powning, R. F., 256
 Praag, D. V., 137
 Pragay, D., 476
 Prange, I., 208, 462
 Pratt, D., 661
 Pratt, E. L., 326
 Preiss, J., 168, 169, 300, 301, 303, 305, 315
 Prescott, D. M., 172, 296, 670, 679, 680
 Prescott, J. F., 136
 Prestidge, L. S., 302, 317, 655
 Presting, W., 73
 Preston, B. N., 662
 Prey, V., 49
 Price, C. C., 58
 Price, J. M., 261
 Price, R. T., 624
 Pricer, W. E., Jr., 175, 196, 232
 Priddle, J. E., 45, 62
 Pridham, J. B., 354
 Prien, E. L., 403
 Prins, H. K., 560
 Prinz, F., 598
 Privett, O. S., 74
 Procházka, Z., 385
 Prosen, R. J., 94, 126
 Proštenik, M., 83
 Prussaf, W. H., 260, 617
 Pryde, E. H., 79
 Przybylska, M., 126
 Puck, T. T., 608, 609, 610, 627, 628
 Pudelskiewicz, W. J., 22
 Pudles, J., 87
 Pullman, A., 647
 Pullman, B., 647
 Pulvertaft, R. J. V., 619, 625
 Pumper, R. W., 610, 624, 627, 628
 Pumphrey, A. M., 19, 22, 459
 Purdom, M. R., 373
 Purves, C. B., 49, 56
 Purvis, J. L., 24, 25
 Putch, J. D., 649
 Putney, R. K., 85, 87
 Pyfrom, H. T., 32
- Q
- Quackenbush, F. W., 541
 Quagliarello, E., 329, 394
 Quastel, J. H., 220
 Quastler, H., 182
 Qureshi, R. U., 327
- R
- Rabate, M. J., 347, 357
 Raben, M. S., 206
 Rabinowitz, J. C., 425, 441
 Rabinowitz, J. L., 208
 Rabson, A. S., 610
 Racker, E., 175, 227, 232, 233
 Radding, C. M., 179
 Rader, Y., 607
 Radermecker, M. A., 340
 Radhakrishnamurthy, R., 412
 Radhakrishnan, A. N., 250
 Radichevitch, I., 372
 Radin, N. S., 72, 74, 79
 Rahman-Li, Y., 34
 Raina, P. N., 250, 607
 Rainbow, C., 348
 Rajagopalan, K. V., 396, 397
 Rajagopalan, R., 337
 Rajalakshmi, S., 393
 Rake, G. W., 609
 Rakowska, M., 538
 Rall, T. W., 138, 225, 226, 260, 362
 Ralph, R. K., 137
 Ramachandran, G. N., 98, 99, 100
 Ramachandran, M., 337
 Ramakrishnan, T. N., 337
 Ramakrishnan, C. V., 250, 607
 Raman, S., 95
 Ramanathan, N. K., 340
 Rama Rao, P. B., 330
 Ramenskaya, G. P., 141
 Ramirez, I., 27
 Rammner, D. H., 49
 Ramos-Galvan, R., 338
 Ramot, B., 232
 Ramstad, E., 544
 Randall, H. M., 85, 87
 Randall, J. T., 99, 100
 Randle, P. J., 221, 233
 Randolph, L. K., 607, 608
 Rands, D. G., 284
 Ranke, B., 402, 404
 Ranke, E., 402, 404
 Rao, B. L., 79
 Rao, D. R., 253
 Rao, K. S., 338
 Rao, N. M., 337
 Rao, P. N., 512
 Rao, S. V., 85, 87, 337
 Rao, T., 330
 Raoul, Y., 456
 Raphael, R. A., 49
 Rapkine, L., 681
 Rapoport, S., 34
 Rapport, M. M., 71, 77, 82, 84, 199
 Rasch, E., 663
 Rasmussen, P. S., 663
 Raut, V., 511
 Ravel, J., 248, 390
 Raw, I., 16
 Rawlinson, W. A., 19, 565
 Ray, A., 452
 Ray, B. L., 26
 Rayroud, A., 463
 Razin, S., 580, 581, 582, 586, 587, 588, 589, 593, 595, 598, 599, 600
 Razumova, L. L., 101
 Razzell, W. E., 140, 141
 Rebel, G., 73, 87
 Recine, A., 457
 Recourt, J. H., 72
 Reddi, K. K., 642, 643
 Redfearn, E. R., 19, 22, 453, 459
 Redman, J., 217
 Redman, W., 629
 Reed, J. M., 29

- Reed, L. J., 35, 36
 Reed, R. I., 46
 Rees, C. W., 50
 Rees, D. A., 63
 Rees, K. R., 26, 29
 Rees, W. R., 351
 Reese, C. B., 50, 136, 138
 Register, U. D., 336
 Reichard, P., 165, 175, 176, 180, 247
 Reichard, S. M., 460
 Reichmann, M. E., 274, 563, 658
 Reichstein, T., 506
 Reid, J. A., 537
 Reid, M. B., 203
 Reid, M. E., 330
 Reinwein, D., 218, 221
 Reisenauer, H. M., 239
 Reiser, R., 190, 191, 192
 Reiss, O. K., 24
 Reist, E. J., 64, 133
 Reithel, E. G., 358
 Reithel, F. J., 358, 364
 Rejnkova, J., 218
 Rekling, E., 449
 Rembarz, G., 51, 56
 Rembold, H., 76
 Remers, W. A., 76
 Remmert, L. F., 23, 594
 Rendi, R., 308, 317
 Rennert, S. S., 334
 Rennie, R., 45, 51
 Renold, A. E., 206, 222
 Repke, K., 511
 Reshef, L., 204
 Resnick, H., 280, 282, 283
 Resler, D., 143
 Reynold, D., 50
 Reynaud, J., 560
 Reynolds, D. M., 374
 Reynolds, J. M., 372
 Rhinesmith, H. S., 111, 276, 560, 561
 Rhuland, L. E., 252
 Riabinina, Z. A., 141
 Ribas, I., 75
 Ricci, P., 580, 598
 Rice, E. G., 537
 Rice, R. V., 100
 Rice, S. A., 644, 645, 647, 648
 Rich, A., 93, 97, 98, 99, 116, 117, 118, 119, 121, 158, 171, 560, 650
 Rich, M. A., 617, 618
 Richards, B. M., 674
 Richards, F. M., 279, 280
 Richards, G. N., 54, 55
 Richards, J., 179
 Richards, R. E., 74
 Richards, R. J., 459
 Richert, D. A., 404, 550, 551
 Richfield, P., 615
 Richman, D. J., 225
 Richeer, G., 73, 74, 172
 Rickards, R. W., 543
 Rickenberg, H. V., 220, 223, 358, 612, 622
 Ricketts, C. R., 63
 Ricklis, E., 220
 Ridyard, J. N. A., 11
 Riedel, A., 99
 Rieder, R. V., 355
 Rieder, S. V., 227
 Riehl, A., 28
 Riggs, A., 558, 559
 Riggs, A. E., 276
 Riggs, A. F., 113, 276
 Rihtniemi, S., 433
 Riley, C., 207
 Riley, H. D., Jr., 34, 232, 653
 Riley, M., 297, 298, 314
 Rilling, H. C., 526, 531
 Rimer, D. G., 222, 230
 Rimington, C., 549, 550, 552, 553, 554, 556, 557
 Rimón, A., 232
 Rindi, G., 458
 Rinehart, J. F., 404
 Rinehart, K. L., 74
 Rinfret, A. P., 239
 Ringelmann, E., 195, 202, 388, 389, 526, 528
 Ringler, I., 511
 Ripley, S. H., 296
 Rippa, M., 17
 Rippon, W. P., 334
 Ris, H., 673, 674, 682
 Risetti, W., 580, 597, 598
 Rittenberg, D., 194, 273, 549
 Riveiro, C., 36
 Rixon, R. H., 629
 Rizzoli, C., 615
 Robbins, D. J., 256, 257
 Robbins, P. W., 224, 362, 366, 514
 Roberts, A. H., 393
 Roberts, E. R., 239
 Roberts, I. Z., 293, 308, 309
 Roberts, J. B., 87
 Roberts, J. D., 64
 Roberts, J. M., 506
 Roberts, N. R., 515
 Roberts, P. J. P., 372
 Roberts, R. B., 293, 308, 309
 Roberts, S., 333
 Roberts, W. K., 139
 Robertson, J. H., 94, 126
 Robertson, J. J., 216
 Robertson, J. M., 95, 126
 Robertson, J. S., 631
 Robertson, O. H., 506
 Robin, Y., 489
 Robins, A. B., 645
 Robinson, A., 627, 628
 Robinson, A. R., 560
 Robinson, D. S., 191
 Robinson, E., 273, 294, 562, 563, 564
 Robinson, E. A., 314
 Robinson, F. M., 526
 Robinson, J. P., 226
 Robinson, R., 553
 Roborgh, J. R., 448, 449
 Rocca, E., 477, 491
 Roche, J., 37
 Rodbell, M., 189, 190, 191
 Roddy, F., 433
 Rodenberg, S. D., 672
 Rodgers, C. G., 334
 Rodgers, Q. P., 333
 Rodin, J. O., 48, 56
 Rodnan, G. P., 461
 Rodnight, R., 261
 Rodwell, V. W., 253, 401
 Roe, J. H., 385
 Roessler, W. G., 241
 Roewer, F., 586, 587
 Rogers, E. F., 433
 Rogers, G. E., 101
 Rogers, L. L., 241
 Rohr, O., 503
 Rohrich, M., 458
 Roitman, E., 326
 Roitt, I. M., 619
 Rokkaku, K., 374
 Rolfe, R., 646
 Rollett, J. S., 95, 102
 Romanek, M., 72
 Romanzi, C. A., 8
 Ronald, A. P., 506
 Roosa, R. A., 608, 611, 627
 Rose, A. H., 389
 Rose, G., 192, 205
 Rose, H., 79
 Rose, I. A., 175, 227, 233
 Rose, W. C., 329, 330
 Roseman, S., 142
 Rosemeyer, M. A., 102
 Rosen, F., 404, 451, 515
 Rosen, G. D., 334
 Rosen, M. F., 513
 Rosen, O., 374
 Rosen, S. F., 456
 Rosenbaum, R. M., 681
 Rosenberg, B. H., 158, 643, 645, 646, 648
 Rosenberg, H., 489
 Rosenberg, H. A., 251
 Rosenberg, H. R., 332
 Rosenberg, H. S., 643
 Rosenberg, T., 218
 Rosenblum, C., 435, 436
 Rosenbluth, R., 479
 Rosenfeld, E. L., 356, 373, 374
 Rosenfeld, R. S., 207
 Rosenheck, K., 288
 Rosenheim, O., 584
 Rosenkrantz, H., 21, 511
 Rosenkranz, H. S., 140, 158, 182, 644, 662

- Rosenthal, O., 502, 506
 Rosenthal, S. 30, 333
 Rosenthal, S. M., 579, 580,
 581, 582, 583, 584, 585,
 586, 587, 588, 590, 597,
 598, 599
 Rosenthal, S. R., 619
 Rositer, R. J., 251
 Roslansky, J. D., 672, 676
 Ross, J. E., 241
 Ross, W. A., 79
 Rossi, G., 385
 Rossi-Fanelli, A., 31, 272,
 274, 275, 562, 563
 Rossiter, R. J., 489
 Rossmann, M. G., 102, 105,
 111, 112, 113, 114, 269, 271,
 559, 560
 Roston, S., 258
 Rotblat, J., 663
 Rotfarb, R. M., 553, 555
 Roth, L. W., 580
 Rothe, M., 459
 Rothfels, K. H., 631
 Rothrock, E., 206
 Rothschild, M. L., 272
 Rothstein, M., 203, 253
 Rotman, B., 215
 Rottenberg, M., 57
 Roulet, F., 587
 Roush, A. H., 177
 Rousseau, J. E., 449
 Roussos, G., 240
 Roux, J., 221
 Rowe, A. J., 477
 Rowse, E. V., 248
 Roy, J. K., 261
 Roychowdhury, S. P., 336
 Rozansky, R., 580, 581,
 582, 586, 593, 595, 596,
 598, 599, 600
 Ruben, S., 556
 Rubin, B. L., 509
 Rubino, A., 329, 394
 Rucker, W., 615
 Rudakova, N. K., 373
 Ruddle, F. H., 627, 630
 Rudloff, V., 275, 561
 Rudman, D., 203
 Rudner, R., 660
 Rudney, H., 460, 526, 543
 Rueckert, R. R., 611, 615
 Riegg, J. C., 477, 484, 485
 Riegg, R., 463, 542
 Rugo, H. J., 101
 Ruiz-Amil, M., 226, 228
 Rummen, N. M., 276
 Rupp, J. C., 477
 Rushinsky, G. W., 642
 Russell, A. E., 535
 Russell, C. S., 550, 551,
 552, 554
 Russi, S., 397
 Rust, J. H., Jr., 615, 616
 Rustad, R. C., 172, 683
 Rutberg, L., 8, 175
 Rutherford, D., 65
 Ruthmann, A., 672
 Ruthven, C. R. J., 260
 Ruzicka, L., 503, 533, 544
 Ryan, K. J., 502, 508, 511
 Rydon, H. N., 259
 Rydzziel, I. J., 30
 Ryhage, R., 72, 74, 75, 87
 Ryle, A. P., 102, 277
 Rysina, T. N., 141
 Ryzasko, C. Z., 484

 S
 Saad, F., 140, 484, 485,
 486
 Saba, N., 501, 504
 Sabina, L. R., 612
 Sable, H. Z., 175
 Sachs, B. A., 537
 Sachs, H., 317
 Sacks, J., 206, 487
 Sacktor, B., 233
 Sadhu, D. P., 403, 452
 Sadron, C., 649
 Sadron, G. A., 649
 Safier, L. B., 254
 Sagan, L. A., 297
 Sagik, B. P., 614
 Sagisaka, S., 253
 Sahashi, J., 454
 Sahashi, Y., 449
 Sahrholz, F. G., 512
 St. George, R. C. C., 271
 St. Lawrence, P., 293, 294,
 295
 Saito, K., 75, 76
 Saito, T., 490, 491
 Saka, T., 581, 598
 Sakai, H., 681
 Sakami, W., 410
 Sakamoto, Y., 402
 Sakan, T., 260
 Sakseia, E., 627
 Salce, L., 366
 Saleminck, C. A., 126
 Salganic, R. I., 141
 Salim, N., 47, 61
 Sallach, H. J., 251
 Salmon, R. J., 182
 Salmon, W. D., 332
 Salomon, L. L., 219, 230
 Salter, J. M., 205
 Saltman, P., 395, 540
 Salton, M. R. J., 364, 374
 Salvatore, G., 245
 Salzman, N. P., 606, 612,
 616, 622
 Salzwedel, M., 79
 Samarina, O. P., 141
 Samejima, T., 32
 Sampson, P., 374
 Samuels, L. T., 505, 507
 Samuelsson, B., 207
 Sanadi, D. R., 26, 27, 28,
 34, 36, 251, 567
 Sanders, E., 189, 190, 191
 Sanders, F., 439
 Sandler, M., 260
 Sandor, T., 503, 505
 Sandritter, W., 679
 Sandy, H. R., 334
 Sands, R. H., 13
 Sanfilippo, A., 355
 Sanford, K. K., 607, 608,
 610, 612, 627, 628, 630
 Sanger, F., 102, 277
 Sankaran, A. N., 337
 Santa Maria, J. V., 336
 Santanam, M. S., 100
 Sapedjinsky, I. I., 141
 Sapryk, V. G., 87
 Saret, L. H., 433
 Sarkar, N. K., 339, 590,
 663
 Sarkar, S., 245
 Sarma, D. S. R., 396
 Sarma, P. S., 393, 396,
 397, 412
 Sartorelli, A. C., 607, 628
 Sasagawa, M., 568
 Sasaki, A. T., 482
 Sasisekharan, J., 100
 Sasisekharan, V., 98, 99
 Saslaw, L., 618
 Satake, K., 663
 Satir, P., 479
 Sato, G., 608, 610, 612,
 626
 Sato, M., 580, 598
 Sato, R., 256, 258, 568
 Sato, T. R., 29
 Sato-Asano, K., 306, 643
 Sautala, H., 677
 Sauer, J., 553
 Saunders, L., 73
 Savage, J. B., 393
 Savard, K., 501, 507, 508,
 511
 Savary, P., 198
 Saville, N. M., 61
 Sawa, Y., 413
 Sawai, T., 355
 Sawyer, E., 550
 Sawyer, T. K., 629
 Saxen, E., 619, 627
 Sayre, D., 126
 Scanlan, J. T., 79
 Scardi, V., 400
 Scarisbrick, R., 568
 Schachinger, L., 26
 Schachman, H. K., 158,
 179, 281, 477, 479
 Schachman, M. K., 646
 Schachter, D., 397, 456
 Schachtschabel, D., 169,
 300, 303, 304
 Schade, H., 395, 540
 Schaechter, M., 671
 Schaefer, P., 659, 660
 Schaeffer, H. J., 133
 Schaffer, R., 56
 Schaffer, M. U., 174
 Schambye, P., 360
 Schanberg, S., 241

AUTHOR INDEX

721

Schapiro, E., 624
 Schaub, R. E., 133, 134
 Schayer, R. W., 254
 Scheijter, A., 33
 Scheinblum, T. S., 474
 Scheler, W., 271
 Schellman, J. A., 281, 283, 285
 Schemer, H. G., 679
 Schendel, H. E., 337, 339
 Scheraga, H. A., 97, 102, 271, 277, 282, 283, 284, 285, 287
 Scherbaum, O., 670
 Scherer, W. F., 632
 Schiff, J. A., 373
 Schildkraut, C., 296, 316, 645, 648
 Schildkraut, C. L., 281, 282
 Schilling, K., 463
 Schimassek, H., 233
 Schindler, R., 617, 618, 624
 Schindler, W. J., 502, 504, 506
 Schirmer, H. K. A., 259
 Schitz, B., 26
 Schlegel, D. E., 658
 Schlenk, F., 256
 Schlenk, H., 71
 Schlesinger, M. J., 526
 Schless, G., 206
 Schlessinger, D., 121, 158, 297, 308, 309, 310
 Schliack, J., 256
 Schliep, H. J., 420
 Schmall, M., 454
 Schmeltz, I., 420, 422
 Schmid, R., 224, 360, 550, 551
 Schmidt, P. J., 506
 Schmidt-Thomé, J., 512
 Schmiechen, R., 56
 Schmitt, E. E., 57
 Schmitt, F. O., 100, 485
 Schmitt, J., 275
 Schneider, J. H., 172
 Schneider, J. J., 514, 597, 600
 Schneider, J. W., 560, 561
 Schneider, K. C., 239
 Schneider, M., 26
 Schneider, W. C., 182
 Schnepf, P., 449
 Schnieden, H., 331
 Schnieder, F., 56
 Schnurbusch, H., 73
 Schoeller, K., 45
 Schofield, K., 50, 138
 Scholander, P. F., 677
 Schole, J., 29
 Scholes, G., 184, 662
 Scholfield, C. R., 74
 Scholtissek, C., 172
 Schomaker, V., 95
 Schon, H., 207

Schönholzer, G., 455, 456
 Schoolar, E. J., Jr., 606
 Schoone, J. C., 104
 Schopfer, W. H., 393, 412, 418, 430
 Schor, R., 101
 Schouten, H., 126
 Schrader, F., 682
 Schramm, G., 123, 298, 364, 375, 560, 561
 Schramm, M., 226, 227
 Schramroth, L., 341
 Schreus, H. T., 598
 Schriefers, H., 510, 512
 Schroeder, W. A., 111, 259, 274, 276, 314, 560, 561, 562, 563, 564
 Schubert, M., 63
 Schuching, S. V., 385
 Schuerch, C., 48
 Schulman, M. P., 404, 550, 551
 Schultz, H. P., 584
 Schultz, J., 30
 Schulz, D. W., 227
 Schulze, H. O., 609, 611, 612, 615
 Schumaker, V. N., 647
 Schuster, H., 661
 Schutz, B., 594
 Schuurs, A. H. W. M., 318
 Schvo, Y., 241
 Schwartz, D., 657
 Schwartz, H. C., 557, 562
 Schwartz, I. R., 654
 Schwartz, K., 21
 Schwartz, M., 333
 Schwartz, R., 339
 Schwartz, S., 435, 551, 553
 Schwarz, H. P., 73
 Schwarz, J. C. P., 47
 Schwarz, K., 421, 460, 461
 Schwarz, R., 45
 Schwarz, V., 220, 231
 Schweet, R. S., 140, 297, 299, 300, 301, 303, 304, 307, 310, 312, 315, 318
 Schweiger, R., 50
 Schweigert, B. S., 327
 Schwert, G. W., 243, 284, 399
 Schwieter, U., 542
 Schwinck, I., 256
 Scoseria, J. L., 36
 Scott, D. B. McN., 671
 Scott, H. M., 331
 Scott, J. F., 140, 244, 299, 300, 301, 303, 307, 310, 515
 Scott, J. J., 252, 550, 551, 555
 Scott, L., 461
 Scott, M. L., 461
 Scott, S. J., 37
 Scott, S. S., 142, 363
 Scotti, T. M., 626
 Scouloudi, H., 104, 110, 270

Scowen, E. F., 251
 Scrimshaw, N. S., 328, 338, 339, 340, 341
 Scult, P. B., 239
 Seaman, G. R., 439
 Searls, R. L., 36
 Sebrell, W. H., 537
 Sebring, E. D., 606, 612, 616, 622
 Secchi, G. C., 333
 Seecof, R. L., 398
 Seeds, W. E., 117, 119, 122
 Segal, H. L., 200, 201, 204
 Segal, S., 231
 Seidman, E. M., 631
 Seidman, F., 203
 Seifter, J., 204
 Seifter, S., 99
 Sekely, L. I., 643
 Sekuzu, I., 12, 13, 19, 564, 568
 Sekuzu, J., 20
 Sela, M., 278, 279, 285
 Seligman, A. M., 24
 Sellinger, O. Z., 34
 Semenenko, G. I., 141
 Senecal, J., 336, 338
 Senko, M. E., 126
 Senkowski, B., 454
 Seno, S., 260
 Senoh, S., 257, 258
 Sensenbrenner, M., 87
 Seoane, E., 75
 Seraydarian, K., 224, 362, 487
 Serck-Hanssen, K., 75
 Serebrennikova, T. P., 476
 Serebnikov, G. P., 141
 Sergeant, T. P., 611
 Sergeev, A. P., 45
 Serif, G. S., 221
 Serman, D., 656, 658
 Servello, L. E., 182
 Sery, T. W., 356
 Seta, M., 280
 Sethi, A. S., 458
 Setlow, J. K., 180
 Setlow, R., 647
 Setlow, R. B., 180
 Seubert, W., 196
 Shafer, E. G. E., 454
 Shafizadeh, F., 133
 Shafir, E., 204, 205
 Shah, P. C., 36
 Shank, R. S., 79
 Shankman, S., 241
 Shannon, J. E., 624
 Shapira, G., 486
 Shapiro, B., 191, 192, 204, 205
 Shapiro, D., 81
 Shapiro, H. S., 178, 295, 642
 Shapiro, P., 143
 Shapiro, R., 50, 138, 330, 332
 Shapiro, S. K., 255

- Sharman, I. M., 450, 459, 462
 Sharon, N., 302, 482
 Sharp, A. G., 552
 Sharpe, E. S., 349
 Sharpe, W. S., 349
 Shatton, J. B., 250
 Shaw, E., 138
 Shaw, G., 136, 137
 Shaw, J. L., 580
 Shaw, K. B., 553
 Shaw, W. N., 204, 222
 Shaw, W. V., 29
 Shearer, H. M. M., 126
 Sheba, C., 232
 Shechter, H., 79
 Sheek, M. R., 614
 Shelton, J. R., 561, 562
 Shemin, D., 273, 435, 549, 550, 551, 552, 554
 Shen, N.-H., 500
 Shen, S. C., 244
 Shengpei-Cheng, S., 141
 Sherman, B. S., 449
 Sherman, F. G., 182
 Shernstev, E. A., 490
 Shibata, K., 274, 563
 Schiffmann, E., 536
 Shiga, T., 37
 Shigeura, H. T., 315
 Shimazono, N., 248, 433
 Shimizu, F., 142
 Shimizu, H., 580, 598
 Shimizu, K., 501
 Shimomura, T., 355
 Shimp, N. F., 12
 Shimura, K., 253
 Shin, M., 564
 Shiner, V. J., 47
 Shiota, T., 423, 424
 Shlyk, A. A., 552
 Shoda, A., 420, 422, 423, 424, 425
 Shoda, T., 420, 423, 424
 Shoemaker, C. B., 94
 Shoemaker, D. P., 95
 Shonk, C. E., 175, 233
 Shooter, K. V., 645, 648, 649
 Shore, P. A., 258
 Shore, V. C., 102, 106, 108, 109, 559, 560
 Shore, V. G., 302
 Shorland, F. B., 72, 75
 Short, R. V., 506
 Shreeve, W. W., 172, 174, 617, 621, 680
 Shubina, A. I., 373
 Shuey, E. W., 363
 Shug, A. L., 658
 Shugaeva, V., 316
 Shugar, D., 150, 154, 156, 157, 284, 662
 Shugar, S., 287
 Shukry, A. S., 340
 Shukuya, R., 242, 243, 399
 Shull, R. L., 462
 Shunk, C. H., 526, 532, 542, 543
 Shuster, L., 144
 Shutte, H. R., 34
 Sibatani, A., 649
 Sideek, M. A., 341
 Sidorova, E., 400
 Sidorova, V. D., 141
 Sidransky, H., 331
 Siegel, A., 661
 Siegel, B. V., 611
 Siegel, S. M., 32
 Sigel, M. M., 626
 Siger, A., 487
 Sih, C. J., 349
 Silberman, R., 85, 259
 Siliprandi, N., 261
 Silk, M. H., 27, 28
 Silva, O. L., 253, 254
 Silver, W. S., 240
 Silverblatt, C. W., 338
 Silverman, M., 253, 426, 587, 592, 599
 Silvers, W. R., 631
 Sim, G. A., 95
 Siminovich, L., 315, 609, 620, 631
 Simkin, J. L., 293, 308, 314, 439
 Simmel, E. B., 174
 Simmons, C., 257
 Simmons, N. S., Jr., 117
 Simms, E. S., 141, 177, 178
 Simon, E., 515
 Simon, E. J., 21, 460, 515
 Simon, H., 64, 420
 Simon, S., 649
 Simons, R. H., 200
 Simpson, J., 76
 Simpson, M. V., 308, 310
 Simpson, W. S., 101
 Simson, P., 663
 Sin, P. M. L., 225
 Sinclair, R., 608, 615, 619, 629
 Singer, F. M., 536
 Singer, M. F., 158, 166, 167, 169, 303, 304
 Singer, S. J., 273, 294, 314, 560, 562, 563
 Singer, T. P., 24, 27, 28
 Singh, H. D., 386
 Singh, R. N., 239
 Sinha, A., 78
 Sinsheimer, R. L., 293, 294, 298, 649, 658, 661
 Siperstein, M. D., 206
 Sirlin, J. L., 173, 174
 Sison, Y., 393
 Sissakian, N. M., 141
 Sistrom, W. R., 214
 Sizer, I. W., 34, 37, 243, 244, 245, 398
 Sjoerdsma, A., 259, 260
 Skank, V., 615, 629
 Skeggs, H. R., 434, 526, 532, 533
 Skertchly, A. R. B., 101
 Skipski, V. P., 71, 77, 84
 Skupp, S. J., 71, 254
 Slade, C. I., 506
 Slater, E. C., 15, 18, 20, 23, 27, 28, 249, 459, 460, 465, 473, 474, 568
 Slater, J. D. H., 514
 Slater, T. F., 23
 Slaughter, C., 252
 Slayter, S. S., 120, 309
 Sleator, W., 483
 Slesinger, M. H., 333
 Slenczka, W., 24, 25
 Sloane, N. H., 255
 Sluysen, M., 169, 311
 Smakula, E., 46
 Smallman, E., 527
 Smellie, F., 329
 Smellie, R. M. S., 168, 170, 179, 180, 618
 Smillie, L. B., 285
 Smith, A. A., 261
 Smith, A. D., 405
 Smith, C. L., 617
 Smith, C. R., 74, 75, 78
 Smith, D. B., 74, 82, 111, 276, 561, 563
 Smith, D. E., 516
 Smith, D. L., 205
 Smith, D. W., 85, 87
 Smith, E. E. B., 363
 Smith, E. L., 287, 435, 557
 Smith, E. N., 477
 Smith, F., 50, 65
 Smith, H., 543, 544
 Smith, H. W., 138
 Smith, J. D., 303, 304, 305, 306, 310, 318, 641, 642
 Smith, J. E. W. L., 97
 Smith, J. K., 536
 Smith, K. C., 140, 307, 317
 Smith, L., 13, 14, 567, 568
 Smith, L. C., 461
 Smith, L. F., 102, 277
 Smith, L. L., 511
 Smith, M., 149
 Smith, M. B., 477
 Smith, M. H., 272, 563
 Smith, M. I., 418
 Smith, M. J., 618, 628
 Smith, M. S., 297, 316
 Smith, O. H., 259
 Smith, R. A., 550
 Smith, R. H., 623
 Smith, R. M., 201, 439
 Smith, R. J., 73
 Smith, S., 611
 Smith, T. E., 245, 254
 Smithies, O., 560
 Smithson, C. H., 241
 Smyth, D. H., 220
 Smyth, R. D., 201, 438, 439

AUTHOR INDEX

723

- Sneath, P. H. A., 590, 591
 Snell, E., 590
 Snell, E. E., 86, 241, 250, 393, 401, 418, 421, 591, 592, 598, 608, 616
 Snellman, O., 476, 478
 Snog-Kjaer, A., 208
 Snoswell, A. M., 34, 228
 Snow, N. C., 276
 Snow, N. S., 558
 Snyder, F., 75
 Snyder, S. H., 254
 Snyderman, S. E., 326, 329, 339
 Sobel, A. E., 449, 456
 Sobel, H., 333
 Sober, H. A., 140, 276
 Sobeslavsky, C., 71
 Sobotka, H., 51, 440
 Socolow, E. L., 606
 Sodd, M. A., 360
 Sokolova, T. D., 141
 Solomon, S., 500, 501, 503, 505
 Sols, A., 213, 216, 219, 223, 226, 227, 228, 230
 Som, J. M., 449
 Somers, C. E., 657
 Somers, G. F., 4
 Somerville, R., 297, 316
 S ndergaard, E., 462, 464
 S rbo, B., 199
 S rensen, J. S., 76
 S rensen, N. A., 76
 Sorger-Domenigg, H., 65
 Sorrels, M. F., 190, 191, 192
 Sorum, H., 126
 Sostman, E., 402
 Soule, H. D., 632
 Southam, C. M., 627, 630
 Sowden, J. C., 52, 53, 55
 Soyama, T., 34
 Spackman, D. H., 102, 269, 277
 Spahr, P. F., 303, 304, 305, 309, 310
 Spalla, C., 437
 Spandrio, L., 34
 Sparks, R. A., 126
 Speakman, P. T., 101
 Spector, L., 247
 Spector, R. G., 260
 Spector-Shefer, S., 81
 Spedding, H., 46
 Speer, V. C., 452, 453
 Spell, W. H., Jr., 176
 Spencer, J. M., 511
 Spencer, M., 114, 117
 Spencer, R. R., 64, 133
 Sperber, R. J., 537
 Spicher, G., 5
 Spiegelman, S., 297, 314, 316
 Spindel, W., 676
 Spirin, A. S., 158, 316, 647, 649
 Spitkovsky, D. M., 141
 Spitzer, J. R., 511
 Sporn, M. B., 248
 Sprague, J. M., 434
 Spray, G. H., 253
 Sprinson, D. B., 45, 436
 Sproull, M., 580
 Sprunt, K., 629
 Spruth, H. C., 464
 Spyrides, G. J., 231
 Squibb, R. L., 338
 Squires, R., 309
 Srikanitia, S. G., 394
 Srikantan, T. N., 20
 Srinivasan, M., 341
 Srinivasan, R., 95
 Srinivasan, V., 393
 Stacey, K. A., 644, 648, 662
 Stacey, M., 45, 46, 47, 50, 57, 58, 59, 60, 62, 63, 65, 349, 350, 371
 Stachenko, J., 501, 504
 Stadie, W. C., 204, 222
 Stadtman, E. R., 201, 202, 249, 388, 435, 439
 Staehelin, M., 140
 Stahl, F. W., 646, 662
 Stainer, R. Y., 87
 Stainsby, G., 99
 St llberg-Stenhagen, S., 72, 74, 75, 87
 Stamas, T. A., 625
 Stambaugh, R., 73
 Stamm, W., 79
 Stammer, C. H., 48, 56
 Stamms, W., 79
 Stancer, H. C., 81
 Standard, K. L., 340
 Standert, J., 475
 Stanley, R. G., 543
 Stanley, W. M., 123
 Stanners, C. P., 617
 Stansly, P. G., 643
 Staple, E., 73, 208, 395, 501, 544
 Stare-Smith, R., 658
 Starling, W. W., 584
 Starr, J. L., 168, 169, 303
 Staudinger, H., 16, 17, 502, 509, 511
 Stavholt, K., 76
 Steberl, E. A., 193
 Stedman, E., 663
 Steffensen, D., 663
 Stefko, P. L., 206
 Stegerhoek, L. J., 81
 Stein, A. M., 515
 Stein, E. A., 356, 365, 372, 373
 Stein, W. D., 278, 279
 Stein, W. H., 102, 269, 277, 278, 558
 Stein, Y., 191
 Steinbach, H., 76, 77
 Steinberg, D., 204, 205, 206, 295, 302, 536
 Steiner, R. F., 156, 255
 Steinhart, J., 275, 558
 Steinrauf, L. K., 95, 97, 276
 Stenbridge, V. A., 189, 190, 191
 Stenhagen, E., 72, 74, 75, 79, 87
 Stenhagen, S., 74
 Stent, G., 119
 Stent, G. S., 298, 314, 316, 661, 662
 Stephen, A. M., 50
 Stephen, J. D., 540
 Stephens, J. F., 466
 Stephens, M. L., 517
 Stephens, N., 75
 Stephens, R. W., 47, 48
 Stephenson, J. S., 76
 Stephenson, M. L., 140, 299, 300, 301, 303, 304, 305, 307, 310, 311, 315
 Stern, B. K., 35, 232
 Stern, H., 677, 680, 681
 Stern, J. R., 199, 202, 203, 389, 439
 Stern, W., 49
 Sternberg, S. S., 627, 630
 Stetten, M. R., 355
 Stevens, A., 171
 Stevens, K. M., 623, 624
 Stevens, M. A., 138
 Stevenson, I. H., 360
 Stevenson, M. L., 167, 169
 Stewart, J., 137
 Stewart, J. E., 46
 Stewart, J. M., 535
 Stewart, S. E., 658
 Stich, H. F., 630, 672, 675, 676
 Stinebring, W. R., 631
 Stockell, A., 276
 Stockell, F. R., Jr., 539
 Stodola, F. H., 349
 Stoffel, W., 72, 75, 76, 77, 78
 Stohlman, E. F., 597
 Stoken, L. A., 181, 182
 Stokes, A. R., 119
 Stokes, W. M., 536
 Stokinger, H. E., 539
 Stolzenbach, F. E., 144
 Stone, B. A., 356
 Stone, M. J., 481
 Storey, I. D., 360
 Storiko, K., 411, 414, 420
 Stotz, E., 13, 15, 19, 22, 23, 27, 28, 73, 83, 199, 459, 549, 564, 565
 Strachan, F., 114
 Stracher, A., 281, 285, 478
 Stradjevskaya, N. B., 141
 Strand, A., 51
 Strandberg, B. E., 102, 106, 108, 109, 269, 270,

- 271, 559, 560
 Strangeways, D. H., 453
 Strassman, M., 250
 Straub, F. B., 372
 Strauss, N., 619
 Strecker, H. J., 254, 435
 Streiff, K., 464
 Streisinger, G., 643
 Stretton, A. O. W., 561, 562, 654
 Strickland, E. H., 73, 83
 Stringer, C. S., 350, 369
 Strittmatter, C., 16
 Strittmatter, P., 16, 19
 Strobach, D. R., 53
 Strohmman, R. C., 476
 Strominger, J. L., 142, 359, 363
 Strong, F. M., 418
 Struchkov, V. A., 141
 Struck, J., Jr., 34, 37, 244, 245
 Struman, M. F., 190
 Strumeyer, D. H., 300, 318
 Struthers, M., 615
 Stuart, K. L., 339
 Stübchen-Kirchner, H., 45
 Stulberg, C. S., 626, 627, 630, 632
 Stumpf, P. K., 196
 Sturtevant, J. M., 37, 275, 490, 558
 Stuttgart, G. Z., 598
 Stutz, E., 373
 Su, J. C., 363
 Suarez, M., 336
 Subrahmanyam, V., 337, 338, 341
 Suda, M., 253, 403
 Sudduth, H. C., 25, 34, 201
 Sueoka, N., 158, 646, 655
 Suetina, I. A., 141
 Sugae, K., 372
 Sugawa, T., 403
 Sugawara, S., 355
 Sugihara, J. M., 49
 Sugimura, A. J., 231
 Sugimura, T., 543
 Sugimura, Y., 568
 Sugino, Y., 142
 Sugita, Y., 557
 Sullivan, B. A., 21, 461
 Sullivan, E., 653
 Sulya, L. L., 73
 Sumner, J. B., 4
 Sunakawa, S., 316
 Sund, K. A., 417, 418
 Sundaram, T. K., 396, 397
 Sunders-Rao, R. V. G., 115
 Sundheim, L., 460
 Sundnes, G., 677
 Sunn, D. B., 305
 Suomalainen, H., 433
 Suschny, O., 615
 Susi, H., 74
 Sussman, K. E., 204
 Sutcliffe, K. W., 220, 231
 Sutherland, E. W., 138, 225, 226, 260, 362
 Sutherland, I., 435
 Sutor, D. J., 115, 116
 Sutton, D., 217
 Suzuki, S., 610, 611, 614, 619
 Suzuki, T., 329, 449, 454
 Suzuoki, J., 428, 431
 Svedberg, T., 4
 Sveinsson, S. L., 677
 Svennerholm, L., 81
 Svenson, G., 679
 Svihla, G., 256
 Swaffield, M. N., 606
 Swaminathan, M., 337, 338
 Swann, M. M., 670, 672, 673, 678, 679, 682, 683, 684
 Swanson, M. A., 371
 Swartz, M. N., 24
 Swarup, S., 338
 Sweat, M. L., 503, 505, 510, 511, 512, 618
 Sweeley, C. C., 73, 455
 Swell, L., 207
 Swendseid, M. E., 327, 329
 Swenson, R. T., 562
 Swern, D., 79
 Swick, R. W., 201, 389
 Swift, H., 671
 Swift, R. W., 327
 Swim, H. E., 510, 511, 606, 607, 608, 609, 615, 618, 625, 627, 628
 Swindels, R., 77, 78
 Sych, G., 56
 Sykes, J., 308
 Sykes, P., 434
 Sylven, B., 680
 Synnatschke, G., 420, 421, 423, 425
 Syverton, J. T., 234, 609, 628, 631, 632
 Szabo, L., 51, 144, 147
 Szczepkowski, T. W., 560
 Szent-Gyorgyi, A. G., 101, 473, 474, 475, 478, 485
 Szibalski, W., 660
 Szilágyi, T., 478
 Szilard, L., 293
 Szulmajster, J., 255, 439
 Szumazkovic, J., 261
 Szybalski, W., 618, 628, 629
- T
- Tabone, D., 360
 Tabone, J., 360
 Tabors, C. W., 579, 580, 581, 582, 584, 585, 586, 587, 588, 590, 591, 593, 594, 595, 596, 597, 598, 599
 Tracey, B. C., 278
 Tacquet, A., 33
 Tada, M., 413
 Tagawa, K., 564
 Taggart, J. V., 397
 Tago, H., 598
 Tai, M., 402
 Taira, T., 412
 Tait, G. H., 252
 Tait, J. F., 501, 504
 Tait, S. A., 501, 504
 Takagi, T., 285
 Takahashi, K., 564, 565, 566
 Takahashi, T., 329
 Takahashi, Y., 261
 Takamutsu, K., 450
 Takaoka, T., 607, 610, 611, 614, 615, 627, 628, 630
 Takaoka, T., 630
 Takeda, I., 413, 414, 415, 416, 418, 419
 Takeda, Y., 568
 Takemasa, Y., 598
 Takemori, S., 12, 19, 20
 Taketa, S. T., 222
 Takeuchi, Y., 126
 Takeyama, S., 255, 440
 Takri, M., 536
 Talalay, P., 35, 509, 510, 514, 515, 516
 Tallan, H. H., 461
 Tallen, D., 384
 Talmage, P., 550
 Tamiya, H., 681, 682
 Tamura, G., 536
 Tamura, S., 536
 Tan, P. H., 486
 Tanaka, A., 87
 Tanaka, J., 288
 Tanaka, R., 248, 433
 Tanaka, T., 259, 490, 491
 Tanaka, Y., 65
 Tandier, C. J., 674
 Tanford, C., 275, 282, 284, 558
 Tani, Z., 388
 Taniguchi, S., 240, 568
 Taniuchi, H., 260
 Tannenhaus, S., 246
 Tao, T. C., 485, 486
 Tapley, D. F., 29, 241, 481
 Tappel, A. L., 21, 27, 461, 463
 Tarjan, R., 450
 Tarr, H. L. A., 490, 491
 Tarver, H., 333
 Tarver, M., 302
 Tashiro, M., 260
 Tasker, P. K., 337
 Tate, S. S., 607
 Tatlow, J. C., 61, 62, 66
 Tattire, N. H., 73, 81, 198
 Tatum, E. L., 427, 431, 641

- Tauber, S. A., 402
 Tavormina, P. A., 535
 Tavs, P., 72, 87
 Taylor, C. B., 220
 Taylor, C. E., 338
 Taylor, D. W., 462
 Taylor, E., 608, 614, 616, 618
 Taylor, E. C., 419
 Taylor, E. W., 672
 Taylor, J. C., 97
 Taylor, J. H., 174, 643, 663, 671, 673, 680
 Taylor, J. M., 173
 Taylor, N. F., 64, 65, 66
 Taylor, W., 510
 Taylore, W. R., 221
 Tchen, T. T., 24, 527, 528, 531, 533
 Teale, F. W. J., 272, 563
 Teerl, A. E., 334
 Teeter, H. M., 79
 Tegge, G., 45
 Teichman, R., 449
 Temple, S., 517
 Templeton, D. H., 126
 Tener, G. M., 119, 134, 139, 140, 147
 Ten Ham, E. J., 448, 449
 Tennent, D. M., 537
 Tenow, M., 476
 Teranishi, R., 72
 Terayama, Y., 487
 Terent'ev, A. P., 584
 Terp, P., 448, 455
 Terrone, E. F., 326
 Terul, G., 217
 Tessman, I., 661
 Testanoff, E. R., 203
 Thale, M., 220
 Thayer, S. A., 207
 Theander, O., 49, 50, 51, 53, 56, 349
 Theivagt, J. G., 454
 Theobald, R. S., 45
 Theorell, H., 276, 564, 566
 Theriault, D., 74
 Thiery, P., 671
 Thind, G., 253
 Thoai, N.-V., 37, 489
 Thom, M. B., 19
 Thoma, J. A., 370
 Thomas, B., 459
 Thomas, C. A., 646, 658, 662
 Thomas, G. J., 356
 Thomas, H. J., 133
 Thomas, J., 215
 Thomas, R., 357
 Thomas, W. A., 189
 Thomas, W. J., 608, 612, 613
 Thompson, A., 133
 Thompson, J. L., 356
 Thompson, K. W., 624
 Thompson, M. J., 536
 Thompson, P. J., 543
 Thompson, R. E., 142
 Thompson, R. H. S., 75
 Thompson, R. R., 55
 Thompson, S. Y., 448, 449
 Thomson, A. E. R., 256
 Thomson, D. H., 184, 662
 Thomson, J. F., 29
 Thomson, R. Y., 180, 616, 618, 620, 621
 Thomson, S. T., 327
 Thorell, B., 27, 680
 Thorin, H., 81
 Thorne, C. J. R., 34
 Thornley, M. J., 216
 Threnn, R. H., 142
 Tichy, M., 58
 Tidwell, H. C., 190
 Tieckelmann, H., 433
 Tiedemann, H., 226
 Tietze, A., 201, 202
 Tiews, J., 447, 449
 Till, J. E., 617, 631, 632
 Timell, T. E., 65
 Tinkler, F. H., 448
 Tinoco, I., 155
 Tipper, D. J., 65
 Tipson, R. S., 46, 64
 Tipton, C. L., 355, 357
 Tisdale, H. D., 19, 20
 Tissières, A., 297, 303, 304, 308, 309, 310, 568
 Titani, K., 564, 565, 566
 Titchener, E. B., 28, 192
 Titmuss, D. H. J., 627
 Tiwari, R. D., 78
 Tixier, R., 433
 Tjio, J. H., 627, 628
 Tobias, C. A., 680
 Tobita, T., 372
 Tocantins, L. M., 654
 Todd, A., 50, 138, 139, 144, 146, 147, 435, 437
 Todd, A. R., 57, 133, 136, 143, 144, 146, 147, 431, 435
 Toennies, G., 663
 Tokarskaya-Merenova, V. I., 141
 Tokuko, S., 580, 598
 Tokuyama, K., 257
 Tokuyama, T., 260
 Toldy, L., 62
 Tomarelli, R. M., 326, 404
 Tomchick, R., 254
 Tomizawa, J., 316
 Tomizawa, S., 618, 628
 Tomkins, G. M., 22, 244, 352, 502, 508, 509, 517
 Tomlin, S. G., 100
 Tomlinson, N., 491
 Tongur, V. S., 141, 165
 Tonomura, Y., 479, 480, 482, 490
 Tonzetch, J., 474
 Toohey, J. I., 201, 438, 439, 440
 Topper, Y. J., 231, 515
 Toralba, G. C., 372
 Torres, F. E., 326
 Torriani, A., 351, 374, 655, 660
 Totter, J. R., 36
 Touchstone, J. C., 500, 501, 506
 Träger, L., 176
 Trager, R., 539
 Traisnel, M., 394
 Tramer, Z., 287
 Trams, E. G., 195
 Trapin, K. M., 277, 278
 Traub, A., 591, 616
 Traut, R. R., 224, 362, 366
 Travieso-Cori, A., 490
 Treadwell, C. R., 207
 Treble, D. H., 419
 Treibs, A., 553, 554
 Trenner, N. R., 542
 Trevelyan, W. E., 365
 Triffett, A. C. K., 77
 Trinkhaus, J. P., 625, 631
 Trommel, J., 95
 Troop, R. C., 509, 511, 512
 Trotter, I. F., 97
 Trousof, N., 384
 Trout, D. L., 205
 Trucco, R. E., 384
 Trueblood, K. N., 94, 95, 115, 126
 Truswell, A. S., 328, 337
 Tsao, M. U., 34
 Tsao, T.-C., 484, 485, 486
 Tschapke, H., 454
 Tschudy, D. P., 551
 Ts'o, P. O. P., 309, 310
 Tsou, C. L., 566, 567
 Tsuboi, K. K., 476
 Tsuchitani, T., 87
 Tsuchiya, H. M., 349, 350, 369
 Tsugita, A., 123, 299, 364, 372, 654
 Tsukihara, K., 413
 Tuboi, S., 302
 Tugwell, R. L., 466
 Tukachinsky, S. E., 486
 Tullner, W. W., 623
 Tulloch, A. P., 72, 78
 Tunatsu, M., 374
 Tung, L., 383
 Tuppy, H., 566, 567
 Turano, C., 261
 Turley, J. W., 115
 Turner, A. F., 139, 147
 Turner, D. A., 73, 81
 Turner, J. C., 63
 Turner, J. M., 259
 Turvey, J. R., 63
 Tuttle, S. G., 327, 329
 Tweddie, J. O., 259
 Twombly, G. H., 511, 513
 Tytell, A. A., 607, 608,

609, 610, 612, 613, 614

U

Uchida, H., 316
 Uchida, K., 479
 Uchida, S., 247
 Udenfriend, S., 241, 245,
 257, 258, 259, 260, 550
 Ueda, K., 428, 429,
 431
 Uezumi, N., 581, 598
 Ugarova, T. U., 141
 Ugoleva, N. A., 141
 Uhlig, H., 123
 Ukita, T., 51
 Ulanova, I. P., 385
 Ulbrecht, G., 483
 Ulbrecht, M., 483
 Ullick, S., 501, 505
 Ulmann, A., 372
 Ulrich, F., 474, 510, 511
 Ulstrom, R. A., 512
 Umharger, H. E., 246, 250
 Urata, G., 554, 557
 Urech, H. J., 504, 506,
 510, 513
 Uretz, R. B., 662
 Urquhart, J., 509
 Uryson, S. O., 141, 649
 Utter, M. F., 233, 389
 Uyeki, E. M., 182
 Usan, A., 433

V

Vagelos, P. R., 193
 Vahouny, G. V., 207
 Valberg, L. S., 462
 Valenta, Z., 553
 Valentine, R. C., 243
 Vallari, V., 612
 Vallee, B. L., 365
 van Bommel, A. J., 125, 126
 Vand, V., 94
 Van Deenen, L. L., 81
 Vandemark, P. J., 229, 230
 Van den Bergh, S. G., 28,
 474
 Vandenheuvel, F. A., 72
 Vanderborght, H., 338, 339
 Vanderhaeghe, F., 172, 296
 Vander Wende, C., 12, 13
 Van de Ven, A. M., 301
 Van Dorp, D. A., 450
 Van Dyke, J. G., 662
 Van Dyke, R. A., 449, 451,
 452
 van Eys, I., 228
 Van Eys, J., 430, 435
 Van Gent, C. M., 72
 Van Iderstine, A., 433
 Van Hallie, T. B., 537
 Vanjushin, B. F., 141
 van Lancker, J. L., 183
 Van Loon, E. J., 537
 Van Maanen, E. F., 536

Van Milligan, H., 24
 Vanngard, T., 36
 Vannotti, A., 567
 Van Vunakis, H., 281, 649
 Vanyushin, B. F., 316, 649
 Varadarajan, S., 136
 Varandani, P. T., 453
 Varga, E., 477, 478
 Vargha, L., 62
 Varner, J. E., 249
 Vasilev, S. S., 45
 Vasington, F. D., 459, 460
 Vaughan, G., 60
 Vaughan, J. H., 624
 Vaughan, M., 204, 205, 206,
 295, 302
 Vecera, M., 72
 Veeger, C., 36
 Veiga, L. A., 229, 230
 Veis, A., 100
 Veldstra, L., 460
 Velle, W. M., 511
 Velluz, L., 45
 Venkataraman, P. R., 174,
 232
 Venkataraman, S., 100
 Venkateshan, C., 337
 Venkster, T. V., 141
 Venner, H., 51, 133
 Vennesland, B., 35, 232,
 243, 597, 600
 Venning, E. H., 506
 Veomett, R. C., 662
 Verdrey, R., 120
 Vereschagin, A. G., 72
 Verkade, P. E., 81
 Vermeulen, A., 510
 Vernon, L. P., 568
 Vielmetter, W., 295, 661
 Vignais, P. V., 452
 Vigneron, M., 341
 Vilkas, E., 82
 Villar-Palasi, C., 223, 224,
 225, 227, 230, 362
 Villee, C. A., 34, 221, 244,
 500, 502, 504, 511, 516
 Villee, D. B., 500, 502,
 504
 Vincent, M., 339, 340
 Vincent, M. M., 624
 Vincent, W. S., 316
 Vinijchaikul, K., 173
 Vinograd, J. R., 273, 274,
 309, 314, 561, 562, 563,
 564, 646
 Virchow, W., 334
 Vis, H., 339
 Vischer, E., 506
 Vischer, M. B., 490
 Viswanatha, T., 253
 Viteri, F., 328, 338, 339,
 340
 Vithayathil, P. J., 279
 Vizoso, A. D., 649
 Vizsolyi, J. P., 143, 144
 Vjlstra, J., 157
 Voegtlin, C., 681
 Vogel, C., 34
 Vogel, H. J., 252
 Vogel, J., 431
 Vogel, R. H., 254
 Vogel, W., 550
 Vogt, M., 627, 628, 629,
 830
 Vohra, P., 253
 Volcani, B. E., 201, 401,
 438, 439
 Volk, B. W., 331
 Volker, M., 563
 Volkin, E., 315, 316, 317
 Volz, H., 553
 von der Decken, A., 169
 von Euler, H., 180
 Von Euw, J., 506
 Von Hippel, P. H., 477, 479
 von Münstermann, A. M.,
 507, 512
 von Sallmann, L., 330
 von Sydow, E., 74
 Vozick, M., 479
 Vyshepan, E. D., 400

W

Wachstein, M., 402
 Wacker, A., 176
 Wada, H., 402
 Wada, T., 600
 Wade, A. P., 514
 Waelisch, H., 584, 590
 Wagenknecht, A. C., 82,
 392
 Wagenknecht, C., 34
 Wagle, S. R., 169, 439,
 451
 Wagner, B. M., 629
 Wagner, F., 436
 Wagner, H., 78, 391
 Wagner, R. P., 250, 398,
 657
 Wahman, R. E., 490
 Wailles, P. C., 77
 Wainio, W. W., 12, 13, 14,
 18, 19
 Wajzer, J., 491
 Wakabayashi, K., 434
 Wake, R. G., 286
 Wakid, N. W., 474
 Wakil, S. J., 192, 193, 194,
 195, 389, 486
 Wakin, K. G., 395
 Wakonig, R., 630
 Walaas, E., 222
 Walaas, O., 222
 Wald, G., 448, 450, 451
 Waldman, E., 49
 Waldschmidt, M., 409
 Walker, D. G., 221, 623
 Walker, D. L., 625
 Walker, G. C., 240
 Walker, G. J., 351, 372
 Walker, J. B., 245, 246,
 403
 Walker, P. G., 374

AUTHOR INDEX

727

Wallace, F. A., 647
 Wallace, R. E., 632
 Wallace, W., 326, 333
 Wallace, W. M., 326
 Wallenfels, K., 388, 364,
 367, 370
 Wallenius, G., 549, 560,
 561
 Wallin, I., 433
 Walop, J. N., 375
 Walsh, P., 403
 Walton, E., 48, 56
 Walton, W., 327, 329
 Waltz, H. K., 623
 Walwick, E. R., 139
 Wang, J. H., 14, 556, 559,
 560
 Wang, T.-Y., 479
 Wang Chu, M.-Y., 619
 Warburg, O., 629, 681
 Warburton, F. G., 387
 Ward, D. N., 649
 Ward, J. R., 184, 662
 Ward, R. B., 65
 Ward, R. J., 458, 462
 Wardlaw, A. C., 296
 Warner, R. C., 157, 158,
 308
 Warnock, L. G., 228
 Warrenner, R. N., 136, 137
 Wartofsky, L., 256
 Washington, F. M., 250
 Wasson, G. W., 193, 525
 Watanabe, A., 79, 454
 Watanabe, H., 433
 Watanabe, S., 483, 489
 Watanabe, T., 95
 Watanabe, Y., 250
 Watari, H., 34, 37
 Waterlow, J. C., 326, 327,
 339, 340
 Waters, W. A., 51
 Watkins, W. M., 373
 Watson, C. J., 435, 551,
 553
 Watson, E. J. D., 513
 Watson, J. D., 117, 118, 124,
 177, 180, 308, 309, 310,
 642, 643, 647, 649, 661
 Watson, M., 120
 Watt, P. R., 458
 Wattenberg, L. W., 22
 Wattiaux, R., 34
 Watts, J. H., 329
 Watts, J. W., 174, 617, 620
 Watts, R. W. E., 251
 Wax, J., 51
 Waymouth, C., 609, 610,
 628, 630
 Weaver, M. L., 658
 Weaver, R. H., 580, 581,
 582, 587, 588, 589, 590,
 598
 Webb, J. P. W., 77, 78, 190
 Webb, M., 615, 674
 Webber, J. M., 45, 47, 61
 Weber, A., 483, 489

Weber, D., 580, 598
 Weber, D. R., 232
 Weber, E., 123
 Weber, F., 459, 460, 463
 Weber, G., 223
 Weber, H. H., 473, 483,
 678
 Weber, M. M., 24, 465
 Weber, R., 491
 Weber, R. E., 282
 Weber, R. P., 74
 Weberling, R., 284
 Webster, G. C., 307, 311
 Webster, L. T., Jr., 300,
 301
 Weedon, B. C. L., 79
 Weigel, H., 349, 356
 Weijers, H. A., 220
 Weijlard, J., 428
 Weiler, E., 625
 Weill, G., 275, 563
 Weimann, G., 139
 Wein, J., 24
 Weinberg, A., 612, 615, 628
 Weinberg, E. D., 580, 598
 Weinberger, L. E., 473
 Weinhouse, S., 200, 250,
 251
 Weiser, R., 364, 374
 Weiss, H., 536
 Weiss, J., 184, 662
 Weiss, J. F., 336
 Weiss, L., 610, 625
 Weiss, M. J., 133, 134
 Weiss, P., 214, 218, 626
 Weiss, S. B., 171, 190, 198,
 299
 Weiss, W., 459
 Weissbach, H., 201, 245,
 260, 261, 438, 439, 440
 Weissler, A. M., 204
 Weissman, S. M., 180
 Weiss-Zehavi, M., 240
 Weitkamp, H., 430, 431
 Welch, A. D., 314, 318, 420,
 610, 618, 627, 628
 Weldon, A. S., 125
 Welhelm, G., 457
 Weliky, V. S., 133, 138
 Welling, W., 183
 Wellings, S. R., 611
 Wellington, J. S., 619
 Wellner, D., 37, 245
 Wells, I. C., 560
 Wells, M., 276, 558
 Wells, W. W., 535
 Welsh, H. K., 95
 Wempen, I., 134, 137
 Wen, H. Y., 485, 486
 Wendt, G., 404
 Wenkeova, J., 191, 205
 Went, H. A., 672, 676, 683
 Werbin, H., 207, 502
 Werle, E., 260, 586, 587,
 597, 598
 Wertheimer, S. A., 643
 Werthessen, N. T., 511

Wessely, K., 51
 West, C. D., 507
 West, R. G., 248, 329
 Westfall, B. B., 606, 607,
 610, 611, 612, 614, 615,
 616, 624, 628
 Westheimer, F. H., 434, 435
 Westphal, K., 431
 Westwood, J. C. N., 615,
 627
 Wetlaufer, D. B., 286, 296,
 477
 Wettstein, A., 501, 503,
 504, 506, 507, 510, 513
 Weygand, F., 56, 64, 409,
 420
 Weymouth, F. J., 143, 147
 Whaley, H. S., 74
 Wharton, F. D., 455
 Whatley, F. R., 465
 Wheatley, P. J., 115
 Wheaton, L., 609
 Wheelodon, L. W., 83
 Whelan, W. J., 45, 46, 51,
 65, 351, 355, 371, 372
 Wherry, F., 459
 Whiffen, D. H., 46, 58, 59,
 60
 Whipple, G. H., 556
 Whistler, R. L., 50, 52,
 54, 55, 373
 Whitaker, D. R., 357
 Whitby, L. G., 355
 White, A., 515
 White, A. M., 439
 White, F. G., 435
 White, F. H., Jr., 280,
 295, 296
 White, I. G., 598
 White, J. C., 561
 White, J. E., 203
 White, J. G., 94
 White, J. I., 486
 White, J. W., Jr., 354
 White, P. B., 606, 615
 White, R. G., 86, 624
 Whitehead, R. W., 176, 177,
 631
 Whitehouse, M. W., 73, 208,
 395, 544
 Whiteley, H. R., 425
 Whiteley, T. E., 51
 Whitfield, J. F., 629
 Whitman, B. T., 72
 Whitmore, G. F., 631
 Whitten, W. K., 375
 Wlame, J. M., 244, 247
 Wick, A. N., 221
 Wickens, J. C., 79
 Widmer, C., 22, 567
 Widom, J. M., 285
 Wieder, C. M., 295
 Wiegand, R. G., 404
 Wieland, O., 201
 Wieland, P., 506
 Wieland, T., 34, 144
 Wiener, E., 565, 566

- Wierzchowski, K. L., 154, 157
 Wiesmeyer, H., 216, 231, 350, 364
 Wiest, W. G., 507, 512
 Wiethe, H., 449
 Wigert, H., 51
 Wilbrandt, W., 218
 Wilbur, K. M., 184, 643
 Wild, G. M., 351
 Wildy, P., 617, 673
 Wilkie, J. B., 448, 454, 455
 Wilkins, M. H. F., 117, 119, 120, 121, 122, 158, 647, 663
 Wilkins, M. J., 649
 Wilkins, R. W., 536
 Wilkinson, G. R., 99
 Wilkinson, J. F., 684
 Wilkinson, J. H., 260, 625
 Will, G., 102, 111, 112, 113, 114, 126, 269, 271, 559, 560
 Williams, A. D., 462
 Williams, A. K., 231
 Williams, E. J., 286, 287
 Williams, G. E., 329
 Williams, G. R., 34
 Williams, H. L., 404
 Williams, J. H., 134
 Williams, J. M., 477, 481
 Williams, J. W., 295
 Williams, M. C., 190, 191, 192
 Williams, O. B., 87
 Williams, R. H., 204, 205
 Williams, R. J. P., 241, 271, 276, 425, 549
 Williams, W. J., 612, 624
 Williams-Ashman, H. G., 516, 517
 Williamson, W. P., 539
 Willis, J. L., 543
 Wills, V. G., 326, 327, 340
 Wilson, D. L., 328
 Wilson, H. R., 117, 119, 120, 158, 647, 663
 Wilson, J. N., 611
 Wilson, M. L., 551
 Wilson, P. W., 239, 394
 Wilson, R. A., 61
 Wilson, R. G., 394
 Wilson, R. M., 438, 439
 Wilson, S., 111, 276, 561
 Wilson, T. H., 219
 Wilson, T. L., 74, 75, 78
 Wilson, W. L., 396
 Winderman, S., 234
 Winegrad, A. I., 204
 Wingate, D. L., 220
 Winitz, M., 125, 330
 Winnick, R. E., 490
 Winnick, T., 490
 Winterbottom, R. J., 473
 Wintersberger, F., 78
 Winterstein, A., 463
 Wintrobe, M. M., 557
 Wise, E. M., Jr., 250
 Wise, J. B., 25, 34, 201
 Wiss, O., 448, 449, 452, 459, 460, 463, 464, 542, 543
 Withers, I., 615
 Witherspoon, B. H., 593, 594
 Witkop, B., 245, 253, 257, 258
 Wittenburg, E., 51, 56
 Wittenberg, J. B., 550, 552
 Witter, R. F., 73
 Wittmann, G., 654
 Wittner, M., 681
 Wittreich, P. E., 526
 Witts, L. J., 253
 Wixom, R. L., 250
 Woernley, D. L., 28
 Woessner, J. F., 623
 Wojtczak, A. B., 23
 Wojtczak, L., 23
 Wolbach, R. A., 113, 559
 Wolf, D. E., 533, 542
 Wolf, G., 449, 451, 452, 453
 Wolfe, J. B., 374
 Wolfe, R. G., 358, 364
 Wolfe, R. S., 435, 441
 Wolff, I. A., 74, 75, 78
 Wolfrom, M. L., 51, 133
 Wolleman, M., 487
 Wollenberger, A., 490
 Wollish, E. G., 454
 Wollman, E. L., 643, 653, 659, 660
 Wolochow, H., 87
 Womack, M., 330
 Wong, E. L., 543
 Wong, G. K., 300, 302
 Wong, R. K. L., 206
 Wong, V., 438
 Wood, B. J. B., 348
 Wood, F. C., Jr., 192, 206
 Wood, H. C. S., 413, 415
 Wood, H. G., 201, 225, 229, 360, 389
 Wood, R. C., 427
 Wood, T., 490
 Woodbine, M., 86
 Woodford, F. P., 72
 Woodliff, H. J., 626
 Woods, D. D., 254, 255, 422, 425, 439, 440
 Woods, E. F., 281
 Woods, H. J., 101
 Woods, M. W., 630
 Woods, P. B., 101
 Woods, P. S., 173, 174
 Woods, R. D., 122
 Woodward, D. O., 653, 658
 Woodward, J. W., 663
 Woodward, R. B., 553
 Wollen, J. W., 374
 Woolley, D. W., 418, 535
 Wooton, J. M., 538
 Work, T. S., 300
 Worker, N. A., 449, 458
 Working, J., 453
 Worner, W., 34
 Worrall, R., 62
 Worthington, C. R., 101
 Wortis, S. B., 261
 Woslait, W. D., 23, 464, 465
 Wotiz, H. H., 46, 505, 507, 508, 511
 Wren, J. J., 73, 85, 259
 Wright, A. M., 387
 Wright, E. M., 175
 Wright, L. D., 525, 526, 528, 532, 533, 535, 538, 539, 600
 Wright, R. S., 134
 Wright, W. B., 96
 Wright, W. G., 329
 Wriston, J. C., Jr., 543, 550
 Wróblewski, F., 34, 228, 612
 Wryk, M. A., 626
 Wu, H., 332
 Wu, R., 233, 606, 612, 615, 616
 Wunderlich, J. A., 125
 Würsch, J., 542
 Wustrow, H. J., 483
 Wyckoff, H. W., 94, 105, 107, 270
 Wyld, M. K., 338
 Wyman, J., 271, 275
- Y
- Yagi, K., 34, 37, 165, 245, 258, 413, 480
 Yagi, M., 33
 Yagushiji, E., 568
 Yajima, T., 490
 Yakel, H. L., 97
 Yamada, H., 260, 355
 Yamada, M. A., 615
 Yamafuji, K., 8
 Yamaguchi, K., 385
 Yamaha, T., 360
 Yamamoto, N., 71
 Yamamura, Y., 87
 Yamana, K., 649
 Yamana, S., 46
 Yamanaka, T., 17, 566
 Yamasaki, G., 217
 Yamashita, J., 17, 564
 Yamazaki, I., 32
 Yanari, S., 286, 287
 Yang, J. T., 283
 Yang, S. P., 329
 Yang, W. C., 25, 34
 Yanofsky, C., 259, 293, 294, 295, 641, 651, 652, 658
 Yanovskaya, B. I., 385
 Yao, F.-O., 205
 Yarmolinsky, M. B., 231

AUTHOR INDEX

729

458 Yasui, T., 482
 Yasunobu, K. T., 257, 259
 Yatco-Manzo, E., 433
 Yates, F. E., 509
 Yates, K., 65
 101 Yavit, J., 428, 431
 Yaw, K. E., 411, 418
 464, Ycas, M., 302, 316, 567
 Yefimochkina, E. F., 491
 5, 507, Yeh, S., 438
 Yerganian, G., 627, 630,
 259 631
 Yielding, K. L., 22, 244,
 517
 526, Yokoyama, H., 541
 538, Yonetani, T., 11, 13, 19
 Yoneyama, Y., 557
 Yorke, R. W., 74
 Yoshida, A., 372
 Yoshida, M., 333
 543, Yoshida, T., 454
 Yoshikawa, H., 372, 557
 228, Yoshikawa, S., 403
 Yoshimura, H., 327
 Yoshizawa, T., 451
 Youatt, G., 356
 Young, D. W., 126
 Young, F. G., 221, 233, 242
 25 Young, I. E., 671
 Young, J., 123
 Young, J. M., 627, 630
 105, Young, L., 256
 Young, R. A., 462
 Young, R. J., 46, 47
 Young, R. S., 176, 177,
 440, 441
 Young, S. P., 327

Yount, R. G., 433
 Yphantis, D., 57, 255
 Yuan, C., 528
 Yudkin, J., 393
 Yuen, G. U., 49
 Yung, N. C., 133, 134, 136
 Yunis, A. A., 364
 Yura, T., 656
 Yurev, V. A., 486
 Yurugi, S., 433
 Yushok, W. D., 219
 Yuwiler, A., 260

Z

Zaalishvili, M. M., 479
 Zabin, I., 215, 540, 541
 Zachau, H. G., 144, 301, 318
 Zaiser, E. M., 558
 Zaitseva, G. N., 141
 Zajdela, F., 28
 Zakharova, N. A., 141
 Zakrzewski, S. F., 426
 Zalkin, H., 21, 461, 463
 Zalokar, M., 171, 172
 Zaltzman, P., 259
 Zamecnik, P. C., 140, 167,
 169, 293, 297, 298, 299,
 300, 301, 303, 304, 305, 307,
 308, 310, 311, 312, 315
 Zander, J., 506, 597, 512
 Zanetti, M. E., 537
 Zannoni, V. G., 257, 387
 Zar, L., 457
 Zarafonitis, C. J. D., 204
 Zarnitz, M. L. 358, 364, 373
 Zaroff, L., 612, 626

Zasler, E. M., 275
 Zbarsky, I. B., 141
 Zebe, E., 233
 Zeitlin, P. I., 141
 Zeller, E. A., 245, 587
 Zeller, P., 436
 Zervas, L., 435
 Zeuthen, E., 670, 673,
 677, 680
 Zhakhova, Z. N., 486
 Ziegler, D. M., 18
 Zienius, E. H., 56
 Zillig, W., 169, 300, 303,
 304, 580, 581, 582, 594
 Zimm, B. H., 644
 Zimmerman, A. M., 669,
 675, 684
 Zimmerman, S. B., 177,
 297, 316, 363, 642
 Zinner, H., 51, 56, 62
 Zinoveva, I. P., 486
 Zischka, W., 624
 Ziskind, B. S., 505, 508,
 511
 Zodrow, K., 550
 Zollicoffer, L., 220
 Zottu, S., 233
 Zubay, G., 119, 120, 121,
 124, 184, 303, 309,
 663
 Zuckerkandl, A., 275
 Zueler, W. W., 560
 Zwartouw, H. T., 615
 Zweifel, G., 58
 Zwiep, N., 448
 Zydowo, M., 486, 488,
 491

SUBJECT INDEX

A

- Absorption
 - of amino acids, 241-42
- Acetaldehyde
 - deoxyribose synthesis from, 174-75
- Acetals
 - formation of, 61-63
- Acetate
 - carotene biosynthesis and, 540-42
 - cholesterol biosynthesis and, 525-27
 - coenzyme Q biosynthesis and, 543
 - ergot alkaloid biosynthesis and, 544
 - nicotinic acid and metabolism of, 395-96
 - riboflavin biosynthesis and, 416
- Acetoacetate
 - metabolism of, 199-201
- Acetoin
 - riboflavin biosynthesis and, 419
- Acetolactate
 - synthesis of, 246
- Acetone
 - cholesterol conversion to, 208
- Acetylation
 - of polyamines, 590
 - ribonuclease activity and, 279
- Acetyl coenzyme A
 - carboxylation of, 192-93
 - permeases and, 215
- N-Acetylglucosamine
 - chitin biosynthesis and, 361
 - hyaluronic acid synthesis and, 362
- N-Acetylglutamate
 - carbamyl phosphate synthetase and, 247
- N-Acetylneuraminic acid
 - cytidine-5'-phosphate and, 142
- Achromobacter fischeri
 - polyamines and, 591-92
- Acridine orange
 - oligonucleotide binding by, 155-56
- Actin
 - actomyosin and, 478-79
 - biochemistry of, 475-77
- F-Actin
 - formation of, 475
- G-Actin
 - polymerization of, 475
- Active acetaldehyde
 - structure of, 434
- Active acyl
 - thiazolium compound and, 435
- Active site
 - of glutamic dehydrogenase, 34-35
 - of ribonuclease, 278-81
- Actomyosin
 - biochemistry of, 478-80
- Adaptor hypothesis
 - protein synthesis and, 310-11
- Adenine
 - 1-N-oxide of, 138
 - riboflavin biosynthesis and, 411
 - vitamin B₁₂ and, 437
- Adenine compounds
 - deamination of in muscle, 490-91
- Adenine nucleotides
 - actin and, 476
 - cultured cells and, 622
 - muscle and, 474
- Adenine toxicity
 - cultured cells and, 618
- Adenosine-3'-5'-cyclic phosphate
 - biochemistry of, 138-39
 - serotonin and, 226
- Adenosine diphosphate
 - mitochondria structure and, 26-27
 - muscle contraction and, 486-87
 - respiration control by, 28
 - synthesis of, 143
- Adenosine tetraphosphate
 - actomyosin and, 481
 - synthesis of, 145
- Adenosine triphosphatase
 - mechanism of action, 483-84
 - mitotic apparatus and, 676
 - myosin and, 480-83
- Adenosine triphosphate
 - acetyl coenzyme A carboxylation and, 194
 - actomyosin breakdown and, 478-79
 - alkali hydrolysis of, 141
 - amino acid activation and, 300
 - carbamyl phosphate synthetase and, 247
 - carboxylations and, 388-89
 - cell division and, 679
 - cholesterol biosynthesis and, 538-39
 - chromosome movement and, 682-83
 - deoxyriboside synthesis and, 175
 - internal incorporation of, 170
 - iron transport and, 385-86
 - mevalonic acid metabolism and, 527
 - muscle contraction and, 486-87
 - nicotinamide adenine dinucleotide and, 26
 - nicotinic acid metabolism and, 397
 - phosphatidic acid biosynthesis and, 196
 - propionyl carboxylase and, 202
 - serotonin and, 260
 - soluble ribonucleic acid synthesis and, 167
 - synthesis of, 143
 - thiamine biosynthesis and, 428
- S-Adenosylmethionine
 - methyl activation and, 255
 - spermidine synthesis and, 585-86
- Adenylic acid
 - crystal structure of, 115
- 5'-Adenylic deaminase
 - in muscle, 490-91
- Adenylosuccinase
 - mutations and, 653
- Adenylyl-2'5'-uridine
 - synthesis of, 147
- Adenylyl-3'5'-uridine-3'-phosphate
 - polymerization of, 150
- Adipose tissue
 - carbohydrate transport in, 22-23
 - lipase content of, 191
- Adrenal cortex
 - stimulation of, 138
- Adrenal gland
 - biosynthesis of hormones in, 500-3
- Adrenocorticotrophic hormone
 - lipid metabolism and, 203-4
- Adrenosterone
 - see Steroid hormones
- Agroclavine
 - biosynthesis of, 544
- Alanine

- crystal structure of, 95
cultured cells and, 613
metabolism of 248-49
 β -Alanine
spermine oxidation and, 589
synthesis of, 249
D-Alanine
alanyl ribonucleic acid synthetase and, 302
Albumin
breakdown of, 333
cell culture and, 609-10
kwashiorkor and, 341
spectroscopy of, 284, 286
sulfhydryl groups in, 296
synthesis of, 624
Aldobiouronic acids
synthesis of, 50
Aldolase
action of, 227
mitosis and activity of, 680
Aldose reductases
specificity of, 229
Aldosterone
see Steroid hormones
Alginic acid
alkali degradation of, 55
Alkaline phosphatase
mutations and, 652
Alkaloids
spermidine in, 583-84
x-ray diffraction of, 126
Alloisoleucine
protein synthesis and, 302
Amethopterin
cultured cells and, 628
Amine oxidase
spermine metabolism and, 586
Aminoacetone
biosynthesis of, 252
Amino acid
cell culture and, 607-8
deficiency symptoms, 331
hydroxamate formation, 300-1
imbalance of, 331-32
in lipid, 85
metabolism of, 239-68
cultured cells and, 612-14
decarboxylation of, 242-43
dehydrogenases and, 243-44
nitrogen metabolism, 239-41
oxidases, 245
penetration of, 241-42
regulation of, 245-47
transamination, 242-43
urea formation, 247-48
see individual amino acids
nucleoside esters of, 144
nucleotide anhydrides of, 145-46
protein nutrition and, 327-31
pyridoxal absorption of, 403
sequence of
alteration of in tobacco mosaic virus, 654
of cytochrome-c, 566
deoxyribonucleic acid and, 293-98
in hemoglobin, 275-77, 561
mutations and, 294
protein conformation and, 295-96
in ribonuclease, 277
ribonucleic acid and, 296-317
ribosomes and, 310-14
vitamin B₁₂ and activation of, 439
x-ray diffraction of, 95-97
D-Amino acid
in lipids, 86
Amino acid analogues
protein synthesis and, 302
Amino acid antagonists
cell culture and, 614-15
Amino acid levels
kwashiorkor and, 339
D-Amino acid oxidase
characterization of, 245
L-Amino acid oxidase
biological oxidation and, 37
characterization of, 245
Amino acid uptake
cultured cells and, 621
Aminoaciduria
kwashiorkor and, 339
vitamin E and, 461-62
Aminoacyl esters
reactivity of, 144
Aminoacyl nucleotides
synthesis of, 146
Aminoacyl ribonucleic acid compounds
formation of 299-308
Aminoacyl ribonucleic acid synthetases
action of, 300
 α -Aminoadipic acid
lysine metabolism and, 253
p-Aminobenzoic acid
metabolism of, 421
methionine biosynthesis and, 254-55
 γ -Aminobutyric acid
spermine oxidation and, 589
Aminoimidazole
biotin and metabolism of, 390
 β -Aminoisobutyric acid
kwashiorkor and excretion of, 339
 α -Amino- β -ketoadipic acid
porphyrin biosynthesis and, 550
 δ -Aminolevulinic acid
biosynthesis of, 550
Aminonucleosides
synthesis of, 133
Aminopterin
cultured cells and, 628
4-Aminopterin
riboflavin biosynthesis and, 411-12
2-Aminopyridine
ester hydrolysis by, 57
3-Amino-1,2,4-triazole
catalase inhibition by, 32
riboflavin biosynthesis inhibition by, 418
Ammonia
in muscle, 490-91
nitrogen fixation and, 239
Amylase
glycogen synthesis and, 226
hydrolytic action of, 355
mechanism of, 370
Schardinger dextrin synthesis and, 351-52
 α -Amylase
biochemistry of, 372-73
metal ion content of, 364-65
 β -Amylase
active site of, 370-71
biochemistry of, 373
Amylase A
spectroscopy of, 285
Amylodextrin
mechanism of hydrolysis, 371
Amylo-1-6-glucosidase
action of, 356
Amylomaltase
biochemistry of, 351
Amylopectin
synthesis of, 351-52
Amylose
mechanism of hydrolysis, 371
synthesis of, 351
Amylosucrase
biochemistry of, 350
Anaphase
chromosome movement in, 682
Androgens
biosynthesis of, 507
effects of, 515-16
Androstenedione
see Steroid hormones
Anemia
ascorbic acid deficiency and, 387
kwashiorkor and, 341
Anerobiosis

- cell culture and, 611
cell division and, 679
Anhydrides, mixed
 reactivity of, 144
Anhydrosugars
 hydrogen bonding in, 60
Anhydrovitamin A
 determination of, 447
Anserine
 in muscle, 490
 vitamin E and levels of, 462
Antephase
 cell division and, 678
Antibiotics
 cultured cells and, 619
Antigens
 cell culture and, 632
 H-meromyosin and, 478
Antimony trichloride
 vitamin A determination and, 447
Antimycin
 site of action of, 18-20
Aparajitine
 structure of, 78
L-Apiose
 synthesis of, 56
Apurinic acid
 dye-binding by, 156
 ultraviolet irradiation of, 157
L-Arabinase
 transport of, 221-22
9-D-Arabinofuranosyl
 adenine
 synthesis of, 134
D-Arabinose
 synthesis from glucose, 50
Arginase
 preparation of, 248
Arginine
 actomyosin and, 480
 cell culture and, 608
 protein nutrition and, 329
Argininosuccinic aciduria
 extent of, 248
Aromatic hydroxylation
 model system for, 258
Aromatic oxidations
 biochemistry of, 258-59
Ascites cells
 sugar transport in, 218-19
Ascorbic acid
 aromatic hydroxylation and, 258
 biochemistry of, 383-88
 biosynthesis of, 383-85
 cytochrome b₅ and, 16-17
 determination of, 388
 functions of, 385-87
 metabolism of, 388
 nutrition of, 387-88
 oxidative phosphorylation and, 27-28
 phenylalanine metabolism and, 256-57
 steroid metabolism and, 502
Ascorbigen
 structure of, 385
Asparagine
 N-alkyl derivatives of, 249
 cell culture and, 607
Asparagine metabolism
 cultured cells and, 614
Aspartic acid
 crystal structure of, 95
 metabolism of, 249
 cultured cells and, 614
Aspergillus nidulans
 polyamines and, 59-91
Aster
 synthesis of, 672
Avidin
 biotin inactivation by, 389
8-Azaguanine
 cytoplasmic ribonucleic acids and, 172
 protein synthesis and, 314
8-Azapurines
 synthesis of, 134
- B**
- Bacteria
 cell division in, 669
 lipids of, 87
 polyamines and stabilization of, 592-93
 spermine agglutination of, 600-1
Bacteriophage
 deoxyribonucleic acid from, 646
 mutations and, 294
 polyamines in, 583
 ribonucleic acid synthesis and, 315
 stabilization of, 594
 transforming principle and, 658-59
Barbital
 ascorbic acid synthesis and, 384-85
Barbiturates
 oxidative phosphorylation and, 29
Barium hydroxide
 action on adenosine triphosphate, 141
Benzaldehyde
 polyhydric alcohols, 62
Benzilic acid rearrangement
 carbohydrate chemistry and, 54-55
Benzmalecene
 cholesterol synthesis inhibition by, 536-37
Benzoate
 D-amino acid oxidase inhibition by, 37
Bicarbonate
 cell culture and, 609
Bile acids
 biosynthesis of, 207-8
Biological oxidation
 see Oxidation, biological
Biosynthesis
 cell division and, 679-80
Biotin
 analogues of, 390-91
 biochemistry of, 388-91
 fatty acid synthesis and, 195
 metabolism of, 391
 nicotinic acid metabolism and, 389-90
 propionate carboxylation and, 201-3, 338-89
Biuret
 structure of complex, 97
Blood coagulation
 ascorbic acid and, 387
 nicotinic acid and, 396
 vitamin K and, 465-66
Bone
 vitamin A and, 452-53
 vitamin D effects on, 457
Boron trichloride
 polysaccharide degradation by, 61
Brain
 amino acid penetration into, 241
Branching factor
 action of, 351-52
Bromoacetic acid
 ribonuclease inactivation by, 278-79
5'-Bromo-5'-deoxythymidine
 crystal structure of, 115
5-Bromouracil
 cultured cells and, 617-18
 mutagenesis and, 660
 non-random incorporation of, 178
5-Bromouridylic acid
 polymerization of, 151
Brucella
 lipids of, 87
Bushy stunt virus
 structure of, 124
Butanedione
 riboflavin biosynthesis and, 415-16
Butyryl coenzyme A
 carboxylation of, 203
 fatty acid biosynthesis and, 193
- C**
- Caffeine
 crystal structure of, 115
Calcium
 actomyosin binding of, 480
 cell culture and, 609

mitotic apparatus and, 676-77
 vitamin D and absorption of, 456
 Canavanine
 cultured cells and, 615
 Cancer cells
 cell culture of, 629-31
 Carbamyl phosphate
 metabolism of, 247
 Carbohydrate
 cell culture and, 609
 chemistry of, 45-70
 alkali effects on, 52-57
 conformation nomenclature, 64
 cyclic acetates, 61-63
 deoxy sugars, 51-52
 fluorine derivatives of, 65
 hydrogen bonding of, 57-61
 inversion of configuration of, 64
 irradiation of, 65
 methylation of, 64-65
 miscellaneous topics, 64-66
 oxidation of, 46-51
 physical methods, 45-46
 sulfate esters of, 63-64
 metabolism of, 213-38
 alternative pathways, 228-33
 control of, 233-34
 cultured cells and, 615-16
 glycolysis, 223-28
 miscellaneous, 233-34
 transport of, 213-23, 622
 x-ray diffraction of, 125-26
 Carbon dioxide
 blot function and, 388
 carbamyl phosphate synthetase and, 247
 vitamin B₁₂ and, 441
 Carboxyhemoglobin
 combination with globin, 272
 Carboxyhemoglobin
 subunits of, 273
 Carboxylation
 of pyruvic acid, 233
 Cardiolipin
 chemistry of 82-83
 chromatography of, 73
 Caries
 vitamin B₆ deficiency and, 404
 Carnosine
 in muscle, 490
 Carotene
 conversion to vitamin A, 449
 Carotenoids
 biosynthesis of, 540-42

Cartilage
 cultured cells and, 626
 mineralization of, 456-57
 Casein
 protein nutrition and, 328-29
 Catalase
 biological oxidation and, 32-34
 cell culture and, 611
 function of, 34
 Catecholamines
 biosynthesis of, 257-58
 cultured cells and, 623
 Cations
 sugar absorption and, 220
 Cell culture
 biochemistry of, 605-40
 cancer and, 629-31
 chemical constitution, 606
 metabolism and, 611-19
 miscellaneous, 631-32
 nucleic acid turnover in, 620-21
 nutritional requirements, 606-11
 protein turnover in, 620
 specialized functions in, 622-26
 transport and metabolic pools, 621-22
 variation and mutation, 626-29
 Cell division
 biochemistry of, 669-88
 chromosome cycle and, 673-74
 chromosome movement and, 682-84
 cytokinesis and, 684
 energetics of mitosis and, 677-79
 metabolic status of, 679-82
 mitotic apparatus and, 674-77
 preparation for, 670-71
 synchronization of, 673
 Cell membrane
 protein synthesis and, 308
 Cell wall
 inositol deficiency and, 393
 Cellobiase
 action of, 356-57
 Cellobiose
 x-ray diffraction of, 125
 Cellobiose phosphorylase
 biochemistry of, 349
 Cellulases
 action of, 356-57
 Cellulose
 degradation of, 356-57
 methylation of, 65
 uridine diphosphateglucose and, 361
 Centriole

reproduction of, 671-72
 Centromeres
 cell division and, 671
 L- α -Cephalin
 synthesis of, 80-81
 Cereal
 protein value of, 334
 Cerebrosides
 chemistry of, 83-84
 chromatography of, 73-74
 Cesium chloride
 deoxyribonucleic acid denaturation and, 648
 Chicks
 protein nutrition in, 330-31
 Chitin
 biosynthesis of, 361
 structure of, 126
 Chloramphenicol
 mitotic apparatus synthesis and, 672
 2-Chloroethanol
 ribonuclease in, 282-83
 p-Chloromercuribenzoate
 effect on myosin, 482
 Chlorophyll
 chemical synthesis of, 553
 Chloroplast
 ribonucleic acids synthesis and, 172
 Chlorpromazine
 oxidative phosphorylation and, 29
 Cholesterol
 absorption of, 206-7
 biosynthesis of, 525-40
 acetate to mevalonate, 525-27
 adenosine triphosphate and, 538-39
 inhibition of, 535-37
 interruption of, 537-38
 mevalonate to squalene, 527-33
 nicotinic acid and, 540
 squalene to cholesterol, 533-35
 vanadium and, 539
 inositol and esterification of, 393
 metabolism of, 207-8
 nicotinic acid and serum levels of, 395
 steroid hormones and, 500-4
 vitamin A and synthesis of, 452
 Δ^7 -Cholesterol
 metabolism of, 535
 Choline
 deoxycytidine pyrophosphate derivative of, 142
 x-ray diffraction of, 126

- Cholinesterase**
 cultured cells and, 625
 kwashiorkor and, 339
Chondroitinase
 biochemistry of, 374
Chondroitin sulfate
 ascorbic acid and, 385
 calcium binding by, 457
 cultured cells and, 623
 desulfation of, 63
Chromatin
 ribonucleic acids syn-
 thesis in, 173
Chromatography
 of acceptor ribonucleic
 acid, 307
 of deoxyribonucleic acid,
 646-47
 of fatty acids, 71-73
 of lipids, 73-74
 of meromyosin, 478
 of nucleic acids, 140
 of polyamines, 579-80
 of tocopherols, 458
 of vitamin A, 447-48
 of vitamin D, 454
Chromium trioxide
 carbohydrate oxidation
 by, 49
Chromosome cycle
 cell division and, 673-74
Chromosome movement
 cell division and, 682-84
Chromosomes
 polyamine effect on, 599-
 600
 reproduction of, 670-71
 structure of, 663
Chylomicrons
 stability of, 189
Chymotrypsin
 deoxyribonucleic acid
 size and, 648-49
 spectroscopy of, 286
Cineole
 biosynthesis of, 543
Citrate
 ascorbic acid and metabo-
 lism of, 386
 vitamin D and metabolism
 of, 457
 x-ray diffraction of, 125
Citronellal
 biosynthesis of, 543
Citrulline
 cell culture and, 607
Cobamide coenzymes
 mechanism of action of,
 438-39
Cobamides
 structures of, 437-38
Cocarboxylase
 see Thiamine pyrophos-
 phate
Coding problem
 mutations and, 294-95
Coenzyme A
 cholesterol and nicotinic
 acid competition for, 395
 synthesis of, 143-45
 triglyceride biosynthesis
 and, 198
Coenzyme Q
 biosynthesis of, 542-43
 oxidation of, 22
 see also ubiquinone
Colchicine
 mitotic apparatus and, 677
Collitose
 guanosine diphosphate
 derivative of, 364
Collagen
 ascorbic acid levels and,
 385
 cultured cells and, 623-24
 denaturation of, 100
 structure of, 98-99
Comparative biochemistry
 actomyosin and, 479-80
Contractile system
 localization of components,
 474
Copper
 cytochrome-c-oxidase
 and, 12
Coproporphyrinogen
 biosynthesis of, 555-56
Coprostanol
 formation of, 208
Coronaric acid
 structure of, 78
Cortical hormones
 vitamin A and synthesis
 of, 451
Corticosteroids
 effects of, 514-15
Corticosterone
 see Steroid hormones
Cortisol
 vitamin B₆ deficiency
 and, 404
 see also Steroid hormones
Cortisone
 see Steroid hormones
Cortolone
 see Steroid hormones
Corynebacteria
 lipids of, 87
Countercurrent distribution
 of lipids, 74
Creatine
 in muscle, 489-90
 negative feedback and,
 246
 vitamin E and metabolism
 of, 461
Creatine phosphate
 in muscle, 489-90
Crotonyl coenzyme A
 carboxylation of, 203
Cryptohemin A
 cytochrome-c-oxidase
 and, 15
Crystal structure
 of amino acids, 95
 of nucleic acid components,
 115
Cyanide
 succinic dehydrogenase
 inhibition by, 18
Cyclic oligonucleotides
 synthesis of, 147
Cyclonucleosides
 deoxyriboside synthesis
 and, 175-76
 synthesis of, 136
Cycloserine
 catalase inhibition by, 33
 transaminase inhibition
 by, 400
Cysteine
 cultured cells and, 612-
 13
 metabolism of, 254-56
 pyridoxal reaction with,
 404-5
 thiazole ring biosynthesis
 and, 431
Cystine
 cell culture and, 607
 configuration of, 95, 97
 crystal structure of, 95
 protein nutrition and, 330
 reduction of, 280
 vitamin A and, 453
Cytidine-5'-phosphate
 N-acetylneuraminic acid
 derivative, 142
Cytidine triphosphate
 adenosine triphosphatase
 and, 481
 internal incorporation of,
 170
 phosphatide biosynthesis
 and, 197-98
 soluble ribonucleic acids
 and, 167
Cytidylic acid
 crystal structure of, 115
Cytochrome a
 biological oxidation and,
 11-15
 copper content of, 12
 oxidation of, 14-15
 purification of, 11-12
 spectrum of, 13
Cytochrome a₃
 cytochrome a and, 13
Cytochrome b
 biological oxidation and,
 18-20
Cytochrome b₂
 biological oxidation and,
 17-18
Cytochrome b₅
 biological oxidation and,
 16-17
Cytochrome c
 binding to enzymes of, 567
 biochemistry of, 564-69
 biological oxidation and,

- 15-16
 biosynthesis of, 567
 coenzyme Q oxidation and,
 22
 denaturation of, 565
 extramitochondrial, 28
 glucose absorption and,
 220
 isolation of, 564
 oxidation of, 14-15
 physicochemical studies
 on, 567
 primary structure of,
 566
 Cytochrome c₁
 biological oxidation and,
 20
 Cytochrome-c-oxidase
 aging of, 15
 components of, 13-14
 copper content of, 12
 inhibition of, 14
 paramagnetic resonance
 of, 13
 pH optimum of, 14
 prosthetic group of, 15
 Cytochrome-c-reductase
 vitamin E and, 459
 Cytochrome f
 isolation of, 568
 Cytochromes
 nomenclature of, 568-69
 oxidative phosphorylation
 and, 27-28
 potentials of, 19
 spectral data for, 19
 Cytokinesis
 cell division and, 684
 Cytolipin H
 chemistry of, 84-85
 Cytoplasm
 deoxyribonucleic acids
 in, 296-97
 Cytosine
 glycerol derivatives of,
 297

D

- Deamination
 ribonuclease activity and,
 279
 of tobacco mosaic virus,
 299
 Decarboxylation
 of S-adenosylmethionine,
 585-86
 of amino acids, 242-43
 of mevalonic acid pyro-
 phosphate, 528-29
 Dedifferentiation
 cultured cells and, 625
 7-Dehydrocholesterol
 metabolism of, 535
 Dehydrocorticosterone
 see Steroid hormones
 Dehydroepiandrosterone
 see Steroid hormones
 Dehydrogenases
 amino acid metabolism
 and, 243-44
 heterogeneity of, 34
 spermine effect on, 600
 Dehydroisqualene
 metabolism of, 531
 Denaturation
 of cytochrome c, 565
 of deoxyribonucleic acids,
 647-49
 of glutamic dehydrogenase,
 35
 of hemoglobin, 275
 Deoxyadenosine
 synthesis of, 51, 133-35
 Deoxyadenyl-5':3'-thy-
 midine
 synthesis of, 149
 Deoxyadenyl-5':3'-thy-
 midine-5'-phosphate
 synthesis of, 149
 Deoxycholic acid
 metabolism of, 207
 Deoxycorticosterone
 see Steroid hormones
 11-Deoxycortisol
 see Steroid hormones
 Deoxycytidine
 synthesis of, 133
 Deoxycytidine diphosphate
 choline
 isolation of, 142
 Deoxycytidine-5-phosphate
 biosynthesis of, 175
 Deoxycytidylate deaminase
 phage infection and, 316
 Deoxycytidyl-5':3'-thy-
 midine
 synthesis of, 149
 2-Deoxyglucose
 cultured cells and, 615
 inhibitory action of, 219,
 228
 periodate oxidation of, 48
 3-Deoxyglucose
 synthesis of, 51
 5-Deoxyglucose
 synthesis of, 51
 6-Deoxyglucose
 synthesis of, 51
 Deoxyguanosine
 synthesis of, 133
 Deoxyhexoses
 in lipids, 86
 Deoxynucleosides
 synthesis of, 133
 Deoxypyridoxine
 bone-graft rejection and,
 403
 Deoxyribonuclease
 oligonucleotide formation
 and, 642
 protein synthesis inhibi-
 tion and, 297
 spermine inhibition of, 600
 Deoxyribonucleic acid
 amino acid sequence and,
 293-98
 antigenicity of, 649
 biosynthesis of, 174-84
 cell content of, 606
 coding problem, 651
 denaturation of, 647-49
 density and base composi-
 tion of, 158
 diphenylamine degradation
 of, 642
 extranuclear, 296-97
 fragility of, 643-44
 genes and, 641
 mutagenesis and, 660
 mutations and, 651
 nearest neighbors in,
 177-78, 643
 nucleotide sequence in,
 295, 641-43
 polyamines and, 595
 preparation of, 649
 radiation damage to, 661-
 63
 renaturation of, 296, 648
 replication of, 650
 ribonucleic acid hybrid
 with, 171, 316
 sedimentation of, 646-47
 shear degradation of, 158
 size of, 643-47
 spectroscopy of, 647
 synthesis of
 in bacteria, 671
 cell division and, 670-
 71
 cultured cells and, 617
 inhibition of, 180
 ionizing radiation and,
 180-81
 turnover of, 620-21
 ultraviolet irradiation of,
 157
 uptake of, 622
 x-ray diffraction of,
 117-18
 Deoxyribonucleic acid
 polymerase
 limited reaction of, 178-
 79
 mechanism of, 180
 phage infection and, 316
 Deoxyribonucleotides
 origin of, 174-77
 Deoxyribose
 biosynthesis of, 174-75
 synthesis of, 51
 Deoxyribose aldolase
 action of, 232
 Deoxyribose polynucleotide
 cytochrome b₂ and, 17
 Deoxy-sugars
 chemistry of, 51-52
 Desmosterol
 metabolism of, 535
 Detergents

- cytochrome a purification
and, 11-12
- oxidative phosphorylation
and, 28
- Deuterium exchange
protein structure and, 271
- ribonuclease and, 281
- Deuterium oxide
cell culture and, 611
- Deuterohemin
peroxidase and, 563
- Dextran
cell culture and, 610
- characterization of, 350
- degradation of, 356
- synthesis of, 371
- Dextranases
action of, 356
- Dextranextrinase
action of, 352
- Dextranucrase
acceptor specificity of,
368
- active site of, 371
- biochemistry of, 349-50
- transacylation and, 347
- Dextran sulfate
desulfation of, 63
- Dextrins
inclusion compounds of,
57
- Diabetes
ketosis in, 200-1
- lipid metabolism and,
205-6
- Diacetylmephermine
in microorganisms, 582
- Dialysis
of lipids, 74-75
- Diamine oxidase
purification of, 245
- 1,4-Diaminobutane
distribution of, 580-84
- spermine and spermidine
and, 579
- 2,5-Diamino-4-mercapto-6-
methylpyrimidine
crystal structure, 115
- 1,5-Diaminopentane
distribution of, 580-84
- Diaminopimelate
lysine biosynthesis from,
252
- 1,3-Diaminopropane
distribution of, 580-84
- 2,6-Diaminopurine
cultured cells and, 628-29
- Diaphragm
carbohydrate transport
and, 222
- p-Diazobenzenesulfonic
acid
cytochrome c coupling
with, 565
- Dicarboxylic acids
x-ray diffraction of, 126
- Dicumarol
oxidative phosphorylation
and, 23
- Dicyclohexylcarbodiimide
nucleotide anhydride syn-
thesis and, 142-43
- polynucleotide synthesis
and, 147
- Diesterases
nucleic acid sequence
studies and, 140-41
- Diet
protein nutrition and, 333-
37
- Diethylstilbestrol
sugar transport inhibition
by, 218
- Digitonin
mitotic apparatus isolation
with, 675
- Digitoxigenin
biosynthesis of, 544
- Diglyceride
phosphorylation of, 196
- Dihydroaldosterone
see Steroid hormones
- Dihydrocortisol
see Steroid hormones
- Dihydrocortisone
see Steroid hormones
- Dihydrofollic acid
metabolism of, 425
- Dihydrolanosterol
metabolism of, 535
- Dihydroprednisone
see Steroid hormones
- Dihydroprogesterone
see Steroid hormones
- Dihydropyran
nucleoside esters of, 149
- 2,4-Dihydroxy-8-ribitylpter-
idine
synthesis of, 413
- Diketopiperazine
planarity of, 96
- x-ray diffraction of, 106
- Dimethylallyl pyrophosphate
metabolism of, 529
- 5,6-Dimethylbenzimidazole
vitamin B12 and, 436-37
- Dimethylethanolamine
existence of, 81
- N,N-Dimethylformamide
periodate oxidation in, 46
- Dimorphecolic acid
structure of, 78
- Dinitrophenol
adenosine triphosphatase
and, 481
- phosphorylation inhibition
by, 23
- Dinucleoside monophosphates
synthesis of, 147
- Dinucleotides
hypochromicity and, 154
- synthesis of, 146-49
- Diphenylamine
deoxyribonucleic acids
degradation by, 642
- Diphenyl-p-phenylene-
diamine
biological effect of, 402
- Diphosphoinositide
in brain, 391
- 5-Diphosphomevalonic
anhydronecarboxylase
action of, 528-29
- Diphosphopyridine nucleo-
tide
see Nicotinamide
- adenine dinucleotide
- Diphtheria toxin
cultured cells and, 619
- Dipyrrylmethene
porphyrin synthesis and,
552
- Dithymidylc acid
synthesis of, 147
- Dobichol
relation to coenzyme Q,
542
- Dopamine
metabolism of, 257-58
- Drosophilin
structure of, 76
- Drug resistance
cultured cells and, 628-
29
- Dyes
oligonucleotide binding of,
155-56
- Dystrophy
vitamin E and, 461-62
- E
- Elastin
structure of, 100
- Electron microscopy
cytokinesis and, 684
- Electron transport
vitamin E and, 459-60
- Electrophoresis
of myogen, 473
- Elymoclavine
biosynthesis of, 544
- Endoplasmic reticulum
cell division and, 680
- lipid absorption and,
189-90
- Engery reservoir
cell division and, 672-73,
678-79
- Enucleate cells
protein synthesis in, 296
- ribonucleic acid syn-
thesis in, 172
- Enzymes
cultured cells and, 611-12
- deoxyribonucleic acid
biosynthesis and, 177-
78
- D-enzyme
action of, 351
- Q-enzyme

- characterization of, 351-52
- R-enzyme
action of, 356
- T-enzyme
action of, 355
- kwashiorkor and, 339
- mitotic apparatus and, 676
- steroid effects on, 517-18
- Epiestriol
see Steroid hormones
- Epimers
of methyl glycosides, 49-50
- Epinephrine
demethylation of, 258
- lipid metabolism and, 204-5
- Episomes
genes and, 659-60
- Episulfides
of carbohydrates, 51
- Epithelial cells
cultured cells and, 626
- Epoxy acids
reactions of, 79
- Ergot alkaloids
biosynthesis of, 544
- Erythrocytes
carbohydrate transport in, 218
- D-Erythrose
ethylidene derivatives of, 62
- Erythrose-4-phosphate
phosphohexose isomerase
inhibition by, 226
- Escherichia coli
carbohydrate transport
in, 214-16
- lipids of, 87
- polyamines in, 582
- Estradiol
amino acid penetration
and, 241-42
- see Steroid hormones
- Estriol
see Steroid hormones
- Estrogens
cultured cells and, 619
- effects of, 516-17
- Estrone
see Steroid hormones
- Ethanolamine
analysis for, 75
- kwashiorkor and excretion
of, 339-40
- Ethionine
protein synthesis and, 302
- Ethoxyquin
antioxidant activity of, 462
- Ethylendiaminetetraacetate
ascorbic acid and iron
chelates, 386
- N-Ethylmaleimide
sulfhydryl reaction with, 276
- Ethyl metaphosphoric acid
isopropylidene derivatives
and, 62
- Ethyl methyl sulfonates
mutagenesis and, 661
- F
- Factor B
structure of, 436
- Factor V_{1a}
structure of, 436
- Farnesinic acid
biosynthesis of, 531-32
- Farnesyl pyrophosphate
metabolism of, 530-31
- Farnesyl synthetase
action of, 531
- Fatty acids
biosynthesis of, 192-96
- chemistry of, 75-79
- cholesterol esters of, 207
- chromatography of, 71-73
- countercurrent distribution of, 74
- hormones and metabolism
of, 203-6
- interconversions of, 196
- insulin and metabolism of, 205-6
- interconversions of, 196
- mass spectrography of, 74
- oxidative phosphorylation
and, 23
- structural determinations
of, 79
- structures of, 76-7
- synthesis of, 79
- vitamin E antagonism of, 462
- Feather rachis
structure of, 101
- Felinine, biosynthesis of, 543
- Ferritin
ascorbic acid function and, 385-88
- Fetuin, cell culture and, 610
- Fibers
x-ray diffraction of, 94
- Fibroblasts
cultured cells and, 626
- galactose transport in, 223
- Fibrous proteins
x-ray diffraction of, 98-102
- Fish flour
nutritional value of, 335
- Flavin
biosynthesis of, 409-27
- succinic dehydrogenase
and, 24
- Flavin adenine dinucleotide
binding sites of, 37
- free radicals and, 36
- succinic dehydrogenase
and, 24
- synthesis of, 143
- tetrahydrofolic acid
metabolism and, 426
- Flavin nucleotides
oxidative phosphorylation
and, 26
- Fluoroacetic acid
cholesterol synthesis
inhibition by, 536
- Fluoroboric acid
catalytic action of, 64
- Fluorodeoxyuridine
cultured cells and, 617
- Fluoromevalonic acid
cholesterol synthesis
inhibition by, 536
- 5-Fluorouracil
cell division and, 671
- protein synthesis and, 314-15
- Fodder
vitamin A content of, 448
- Folic acid
biochemistry of, 409-27
- cell culture and, 608
- riboflavin biosynthesis
and, 412
- vitamin B₁₂ and, 440
- Folic acid reductase
characterization of, 426
- Foods
protein rich, 335-37
- Formaldehyde
vitamin B₁₂ and, 440-41
- Formate
riboflavin biosynthesis
and, 410
- vitamin B₁₂ and, 440-41
- Formiminoglutamic acid
histidine metabolism and, 253
- Free radicals
peroxidase activity and, 31-32
- xanthine oxidase and, 36
- Fructosans
formation of, 352-53
- Fructose
intestinal metabolism of, 219-20
- metabolism of, 230
- placenta and, 221
- Fructose diphosphate
cell culture and, 609
- Fructose-1-phosphate
metabolism of, 230
- α -L-Fucosidase
biochemistry of, 373
- G
- Galactokinase
specificity of, 231
- Galactose
alkali degradation of, 52
- cell culture and, 609

- glucuronic acid pathway
and, 384
kidney absorption of,
220-22
lipid incorporation of, 360
metabolism of, 231
transport of, 215-16
uptake of, 622
Galactose-4-sulfate
infrared absorption of,
63-64
 α -Galactosidase
action of, 358-59
 β -Galactosidase
action of, 357-58
active site of, 370
carbohydrate content of,
364
deoxyribonucleic acid
and synthesis of, 297
mutations and, 294
regulatory mutations and,
654-55
Galactosides
transport of, 214-15
Gangliosides
chemistry of, 84
Gas chromatography
of carbohydrates, 45
of fatty acids, 72
Gaucher's disease
cerebrosides and, 63-84
Gelatin
protein value of, 335
Gene
complementation of,
657-58
deoxyribonucleic acid
and, 641
episomes and, 659-60
linkage of, 656
Genetic factors
biochemistry of, 641-68
Genetic information
expression of, 650-60
Genome
protein synthesis and, 298
Geranyl pyrophosphate
metabolism of, 530
Gibberellic acid
biosynthesis of, 543
Globin
preparation of, 272
Globular proteins
x-ray diffraction of, 102-13
Globulin
cell culture, 609-10
Glucagon
lipid metabolism and, 205
 β (1-3) Glucan
biosynthesis of, 361
D-Glucitol
cleavage of derivatives of,
61-62
Glucosamine
vitamin D and synthesis
of, 457
Glucosamine-6-phosphate
cultured cells and, 613
 β -Glucosaminidase
biochemistry of, 374
D-Glucosaminuronic acid
synthesis of, 50
Glucose
alkali rearrangement of, 53
arabinose synthesis from,
50
bromine oxidation of, 50
cell culture and, 609
degradation of, 63
gamma-ray irradiation of,
65
glucuronic acid metabolism
and, 384
intestinal absorption of,
219
leucopterin biosynthesis
and, 420
metabolism and cultured
cells, 615-16
sulfation of, 63
transport of, 216
uptake of, 622
x-ray diffraction of, 125
D-Glucose-6-acetate
preparation of, 65
Glucose-6-phosphatase
mechanism of, 231-32
Glucose-1-phosphate
glycogen synthesis from,
224
Glucose-6-phosphate
active form of, 226-27
glycogen synthetase
activation by, 225
Glucose-6-phosphate de-
hydrogenase
activation of, 232
mutations and, 653
steroid effects on, 517
Glucosidase
biochemistry of, 373
 α -Glucosidase
intestinal, 220
 β -Glucosidase
oligosaccharide synthesis
by, 372
transosylation and, 347
Glucosides
uridine diphosphate sugars
and, 360
Glucosidic bond
bond energy of, 365-66
Glucosido-invertase
action of, 354
 α (1-4) - Glucosylases
action of, 355
 α (1-6) - Glucosylases
action of, 356
Glucuronic acid
hyaluronic acid synthesis
and, 362-63
inositol metabolism and,
393
Glucuronic acid pathway
activation of, 384
 β -Glucuronidase
action of, 359
biochemistry of, 374-75
polyamines and, 595
Glucuronides
biosynthesis of, 360
D-Glucuronolactone
ascorbic acid metabolism
and, 383
Glutamic acid
crystal structure of, 95
metabolism of, 249-50
 β -methylaspartate biosyn-
thesis and, 438
transaminase and, 249
Glutamic acid decarboxylase
characterization of, 399
preparation of, 242-43
Glutamic acid dehydrogen-
ase
biochemistry of, 243-44
biological oxidation and,
34-35
Glutamic semialdehyde
metabolism of, 254
Glutamine
cell culture and, 607
metabolism of, 612
Glutamine synthetase
cultured cells and, 612
reactions of, 249-50
Glutaryl coenzyme A
metabolism of, 203
Glutathione
biosynthesis of, 300
mitosis and, 691
polyamine derivations of,
590
vitamin B₆ deficiency
and, 405
x-ray diffraction of, 95-
96
Glutathione peroxidase
role of, 30
Glutathione synthetase
ribonucleic acid binding
to, 307-8
Gluten
protein value of, 335
Glycals
epoxidation of, 61
Glyceraldehyde
alkali rearrangements
of, 53
Glyceraldehyde-3-phosphate
deoxyribose synthesis
and, 174-75
Glyceraldehyde-3-phosphate
dehydrogenase
inhibition of, 227-28
mitosis and activity of,
680
Glycerides
chromatography of, 73
Glycerol

- cytosine and uracil derivatives of, 297
 - lipid absorption and, 190
 - L- α -Glycerophosphate phosphatide precursor, 197
 - α -Glycerophosphate dehydrogenase
 - significance of, 233
 - thiazole cofactor for, 435
 - Glycerophosphate pathway
 - recent developments in, 233
 - Glycine
 - collagen and, 99
 - crystal structure of, 95
 - cultured cells and, 613
 - metabolism of, 250-51
 - porphyrin biosynthesis and, 549-50
 - vitamin E and levels of, 461
 - Glycogen
 - cultured cells and, 616
 - metabolism of, 223-26
 - uridine diphosphoglucose and biosynthesis of, 362
 - vitamin A and synthesis of, 451
 - vitamin E and levels of, 461
 - Glycogen synthetase
 - purification of, 224-25
 - Glycolipids
 - synthesis of, 81
 - Glycolic acid
 - irradiation of, 65
 - Glycolipids
 - chemistry of, 83-85
 - Glycolysis
 - biochemistry of, 223-28
 - cultured cells and, 615
 - mitosis and, 681
 - Glycosidases
 - biochemistry of, 347-82
 - Glycosides
 - alkali stability of, 55-56
 - Glycosyl purines
 - synthesis of, 133
 - Glycosylpyrimidines
 - synthesis of, 134
 - Granuloma
 - proline metabolism and, 254
 - Growth
 - cell division and, 670
 - Growth hormone
 - lipid metabolism and, 203-4
 - Growth inhibitors
 - cultured cells and, 618-19
 - Guanosine diphosphate
 - polymerization of, 166
 - ribonucleic acid end group and, 169
 - sugar derivatives of, 142
 - vitamin B₁₂ and, 437
 - Guanosine diphosphate mannose
 - mannan synthesis and, 364
 - Guanosine diphosphate sugars
 - isolation of, 363
 - Guanosine triphosphate
 - hemoglobin synthesis and, 312
 - Guanylic acid
 - nucleotide sequence and, 168
 - L-Gulonic acid
 - metabolism of, 383
 - Gut
 - albumin breakdown in, 333
- H
- HeLa cells
 - macromolecule content of, 600
 - Helix
 - in polyribonucleotides, 118-19
 - x-ray diffraction of, 94, 98
 - α -Helix
 - in myoglobin, 270-71
 - in ribonuclease, 277
 - β -Helix
 - myosin and, 477
 - Heme
 - biosynthesis of, 556-57
 - heme-heme interactions, 271-72
 - hemoglobin location of, 12, 271
 - linkage to protein, 271-72
 - myoglobin location of, 106
 - vitamin B₉ of, 404
 - Heme synthetase
 - preparation of, 557
 - Hemin
 - cytochrome b₅ and, 16
 - dissociation of, 562
 - Hemin a
 - cytochrome a and, 15
 - Hemoglobin
 - abnormal types of, 560
 - amino acid alterations in, 653
 - amino acid sequence in, 111, 275-77
 - biochemistry of, 557-64
 - biosynthesis of, 557
 - cell free synthesis of, 312
 - conformation of, 270-73
 - denaturation of, 275
 - dissociations and recombinations, 273-75, 562-64
 - heterogeneity of, 276-77
 - hybridization of, 273, 563
 - isomorphous replacement and, 104
 - physicochemical studies on, 558-60
 - primary structure of, 560-62
 - structure of, 110-13
 - sulfhydryl groups in, 276
 - x-ray diffraction of, 271
 - Hemoglobin A
 - structure of, 562
 - Hemoglobin A₂
 - structure of, 562
 - Hemoglobin, Bart's
 - structure of, 562
 - Hemoglobin F
 - structure of, 562
 - Hemoglobin H
 - structure of, 562
 - Hemophilus parainfluenzae
 - polyamines and, 590
 - Hemoproteins
 - biochemistry of, 557-78
 - Heparin
 - desulfation of, 63
 - lipase activation of, 191
 - polyamine binding by, 597
 - Hexokinase
 - biochemistry of, 226
 - Hexose monophosphate
 - shunt see Pentose phosphate pathway
 - Hexose-6-sulfate
 - infrared absorption of, 63
 - Histamine
 - cultured cells and, 624
 - metabolism of, 254
 - spermine and, 597-98
 - Histidine
 - amino acid imbalance and, 332
 - crystal structure of, 95
 - hemoglobin denaturation and, 275
 - iodoacetate reaction with, 278
 - iron binding to, 27
 - metabolism of, 253-54
 - position in myoglobin of, 107
 - protein nutrition and, 330
 - Histidine decarboxylase
 - pyridoxal phosphate and, 398-99
 - Histones
 - function of, 663
 - Histone synthesis
 - cell division and, 671
 - Holothurin A
 - 6-deoxy-glucose in, 51
 - Homogentisic acid
 - melanin formation from, 259
 - Homogentisicase
 - characterization of, 257
 - Homografts
 - deoxyypyridoxine and rejection of, 403
 - Hookworm

- protein nutrition and, 333
- Hormones**
 adrenal, 500-3
 amino acid penetration and, 241-42
 biochemistry of steroids and, 499-524
 carbohydrate metabolism and, 233
 cell culture and, 611
 fatty acid metabolism and, 203-6
 glutamic dehydrogenase and, 244
 ovarian, 503-7
 placental, 508
 testicular, 507-8
 see also Steroid hormones
- Humans**
 protein nutrition, 326-30
- Hyaluronic acid**
 cultured cells and, 623
 uridine diphosphate sugars and, 362-63
- Hyaluronidases**
 biochemistry of, 373-74
 polyamine effect on, 600
- Hybridization**
 of hemoglobin, 273
- Hydrocortisone**
 cultured cells and, 619
- Hydrogen bonding**
 acceptor ribonucleic acid and, 170
 carbohydrates and, 57-61
 deoxyribonucleic acid denaturation and, 648
 mitotic apparatus and, 676
 nucleic acids and, 114-15
 polynucleotide phosphorylase and, 167
 protein spectroscopy and, 284
 ribonuclease titration and, 283-84
 in ribonucleic acid, 649-50
 ribosomal ribonucleic acid and, 309-10
- Hydrogen peroxide**
 ascorbic acid synthesis and, 383-84
 glutathione peroxidase and, 30
- Hydrogen transfer**
 from cytoplasm, 233
- Hydrolases**
 donor specificity of, 368
- Hydrophobic bonds**
 protein structure and, 288
- β -Hydroxybutyrate dehydrogenase**
 mitochondria and, 25
 properties of, 201
- α -Hydroxyethylthiamine**
 active acetaldehyde and, 434
- Hydroxylamine**
 oxidation of, 240-41
 reduction of, 240
- Hydroxylation**
 of aromatic compounds, 258
 ascorbic acid and, 386
 of steroids, 512-14
- Hydroxyllysine**
 metabolism of, 253
- Hydroxymethylase**
 phage infection and, 316
- Hydroxymethylcytosine**
 glucosylation of, 363, 642-43
- β -Hydroxy- β -methylglutaric acid**
 mevalonic acid biosynthesis and, 526
- Hydroxymethylglutaryl coenzyme A**
 metabolism of, 200
- 5-Hydroxymethyluridine**
 synthesis of, 137-38
- p-Hydroxyphenylpyruvic acid**
 ascorbic acid function and, 387
 metabolism of, 256-57
- 17-Hydroxyprogesterone**
 see Steroid hormones
- Hydroxyproline**
 crystal structure of, 95
 metabolism of, 254
- Hydroxypyruvate**
 significance of, 251
- Hydroxysteroid dehydrogenase**
 biochemistry of, 509-12
- 5-Hydroxytryptophan**
 decarboxylation of, 260
- Hyperglycemia**
 spermine and, 597
- Hyperoxaluria**
 glycine metabolism and, 251
- Hypochromicity**
 of oligonucleotides, 153-55
 pH effect on, 154
- I
- Imidazolone propionic acid**
 metabolism of, 253
- Inclusion compounds**
 with uridylic acid, 57
- Infants**
 protein requirement of, 326
- Infrared spectroscopy**
 of carbohydrates, 46
 of lipids, 74
- Inosine**
 synthesis of, 136-37
- Inosine monophosphate**
 in muscle, 491
- Inosine triphosphate**
 adenosine triphosphatase and, 481-82
- Inositol**
 analysis for, 75
 biochemistry of, 391-94
 cultured cells and, 616
 metabolism of, 393-94
 phosphates of, 391-93
 protein complexes with, 57
 separation of, 394
- Inositol phosphatides**
 biosynthesis of, 197-98, 392-93
 purification of, 82
 structures of, 391-93
- Insulin**
 action on adipose tissue, 222-23
 cell culture and, 611
 glucose transport and, 221
 lipid metabolism and, 205-6
 production of, 623
 reoxidation of sulfhydryl groups of, 296
 spectroscopy and, 284-85
 structure of, 102
- Interphase**
 cell division and, 670
 deoxyribonucleic acid synthesis in, 671
- Intestine**
 carbohydrate transport in, 219-20
- Intrinsic factor**
 vitamin B₁₂ binding by, 438
- Intrinsic viscosity**
 of deoxyribonucleic acid, 644
- Inulosucrase**
 action of, 353
- Invertase**
 action of, 353-54
 mechanism of, 369-70
 purification of, 364
- Iodoacetamide**
 myosin inactivation by, 482
- Iodoacetic acid**
 ribonuclease inactivation by, 278-79
- Ionizing radiation**
 deoxyribonucleic acid synthesis inhibition by, 180-84
- Iris tissue**
 cultured cells and, 626
- Iron**
 ascorbic acid and, 385-86
 heme formation and, 550, 556-57
 histidine co-ordination to, 27

myoglobin and, 109
transaminase and, 398
Isocaproic acid
cholesterol metabolism
and, 501
Isocitrate
x-ray diffraction of, 125-26
Isoleucine
crystal structure of, 95
metabolism of, 250
protein nutrition and, 328
Isoleucyl-ribonucleic acid
synthetase
specificity of, 301-2
Isomaltose
transport of, 217
Isomaltulose
synthesis of, 349
Isomorphous replacement
x-ray diffraction and,
102-4
Isonicotinic acid hydrazide
enzyme inhibition by, 400
Isooctane
enzyme systems and, 21
vitamin E activity and,
459-60
Isopentenylpyrophosphate
metabolism of, 528-29
Isopentenylpyrophosphate
isomerase
characterization of, 529
Isoprenoid compounds
biosynthesis of, 525-48
Isopropylidene derivatives
of carbohydrates, 62
gas chromatography of, 45
Isosaccharinic acids
synthesis of, 52
Isozymes
definition of, 228

J

Jerusalem artichoke
transfructosylases of, 353

K

Karyotype
of cultured cells, 627
Keratin
structure of, 100-1
vitamin A and, 453
2-Keto-3-deoxy-8-phospho-
octonic acid
synthesis of, 233
 α -Ketoglutarate
biological oxidation and,
35-36
Ketone body
metabolism of, 199-201
Ketose reductases
specificity of, 229
Ketosis
mechanism of, 200-1
Ketosteroid reductases

biochemistry of, 509-12
Kidney
cultured cells from, 625
spermine effect on, 597
sugar transport in, 220
Kwashiorkor
enzyme levels in, 339
protein nutrition and, 337-
41
Kynurenic acid
degradation of, 260-61

L

Laccase
oxygen complex with,
13-14
Lactic dehydrogenase
cytochrome b₂ and, 17-18
varieties of, 228
Lactobacillus casei
polyamines and, 591
Lactones
differentiation of, 46
Lactose
alkali degradation of, 52
cell culture and, 609
dextranucrase action
and, 349
Lactose-phosphate
uridine diphosphate
galactose and synthe-
sis of, 360
Laminarin
mass spectroscopy of, 46
Lanosterol
metabolism of, 533-34
Lard
protein nutrition and, 330
Lead tetraacetate
glycol cleavage by, 47
Leaf protein
nutritional value of, 337
Lecithin
metabolism of, 198-99
polyamine binding by, 597
synthesis of, 79-80
Leucine
crystal structure of, 95
metabolism of, 250
nicotinic acid metabolism
and, 394
protein nutrition and, 330
Leucocytes
carbohydrate transport in,
223
cultured cells and, 626
Leucopterin
biosynthesis of, 409
Leucrose
biosynthesis of, 349
Levans
mechanism of synthesis,
371
Levansucrase
acceptor specificity of,
368
action of, 352-53
donor specificity, 368
equilibrium constant of,
365
mechanism of, 369-70
Light scattering
of deoxyribonucleic acid
solutions, 644-45
Lipids
amino acid containing,
85-86
analytical methods, 71-
75
biological oxidation and,
20-24
chemistry of, 71-92
chromatography of, 73-
74
countercurrent distri-
bution of, 74
detection of, 73
dialysis of, 74-75
fatty acids, 75-79
glycolipids, 83-85
infrared spectrography of,
74
mass spectrometry of,
74
metabolism of, 189-212
biotin and, 390
cholesterol absorption
and, 206-7
fatty acid biosynthesis
and, 192-96
hormone effect on, 203-
6
ketone body and, 199-201
nicotinic acid and, 395-
96
phospholipid biosynthe-
sis and, 196-99
propionic acid and, 201-
3
steroid, 207-8
triglyceride absorption,
189-90
triglyceride metabolism,
190
vitamin B₆ and, 403
of microorganisms,
86-87
mitotic apparatus and,
675
nuclear magnetic reson-
ance of, 74
peptidoglycolipids, 86
phospholipids, 79-83
polyamine binding by,
596-97
Lipoamino acids
chemistry of, 85
Lipoprotein
lipase activity of, 191
Lipoyl dehydrogenase
activity of, 36
Liver
cultured cells and, 626

- triglyceride entry into, 190-91
- Lombricine biosynthesis of, 251
- Lumazines riboflavin biosynthesis and, 412-13
- Lunarine spermidine in, 584
- Lycopene biosynthesis of, 541
- Lycorine ascorbic acid synthesis and, 385
- Lysine amino acid imbalance and, 332
crystal structure of, 95
iodoacetate reaction with, 278
kwashiorkor and, 338
metabolism of, 252-53
protein nutrition and, 328
wheat flour and, 337
- Lysolecithin isolation of, 81-82
- Lysozyme action of, 364
mutation and, 294
spectroscopy of, 286
- L-Lyxonic acid biosynthesis of, 388
- M**
- Magnesium actomyosin binding of, 480
concentration in muscle, 475
- Malignancy cultured cells and, 627
- Malignolipin characterization of, 581
isolation of, 598
- Malonaldehyde ascorbic acid synthesis and, 384
- Malonic acid x-ray diffraction of, 126
- Malonyl coenzyme A fatty acid biosynthesis and, 192-93
- Maltase action of, 354-55
- Maltose alkali degradation of, 54
amylose synthesis from, 351
transport of, 216
- Maltose phosphorylase biochemistry of, 348
donor specificity of, 368
equilibrium constant of, 365
- Maltotriose metabolism of, 351
- Mammals polyamines in, 580-81
- Manganese dioxide carbohydrate degradation by, 50-51
- D-Mannitol cyclic acetal formation from, 61
- Mannose alkali degradation of, 52-53
guanosine diphosphate derivative of, 364
toxicity of, 230-31
- Margarine vitamin A determination in, 448
- Mass spectrometry of carbohydrates, 46
of lipids, 74
- Meat protein value of, 334
- Meat flour nutritional value of, 336
- Melanin cultured cells and, 623
free radicals and, 258
- Melatonin biochemistry of, 261
- Melibiose metabolism of, 358-59
- Menadiene biological activity of, 464
tetrazolium reductase and, 22
- Mercaptoacetic acid unsaturation test, 75
- 6-Mercaptopurine cultured cells and, 618, 629
- Mercuri-acetate fatty acid separation and, 75
- Mercuric chloride oligonucleotide interactions with, 158
- Meromyosin spectroscopy of, 285
subunits of, 477-78
- H-Meromyosin effect on G-actin, 475
localization of, 474
- L-Meromyosin localization of, 474
tropomyosin A and, 485
- Mesohemin globin combination with, 562
- Metabolism cell division and, 679-82
- Metamyosin composition of, 486
- Metasaccharinic acids synthesis of, 52
- Methemoglobin heme interchange in, 273
- Methionine absorption of, 241
crystal structure of, 95
cultured cells and, 612
iodoacetate reaction with, 278
metabolism of, 254-56
protein nutrition and, 328
spermidine biosynthesis and, 585-86
thiazole ring biosynthesis and, 431-32
vitamin B₁₂ biosynthesis and, 435-36
vitamin B₁₂ and synthesis of, 439-40
- Methostenol metabolism of, 535
- N-Methylacetamide x-ray diffraction of, 96
- 9-Methyladenine crystal structure of, 115
- 1-Methyladenylic acid methyl migration and, 138
- 2-Methyl-4-amino-5-hydroxy-methylpyrimidine analogues of, 429-30
thiamine biosynthesis and, 428
- 4-Methyl- Δ^8 -cholesterol metabolism of, 535
- 5-Methylcytosine acceptor ribonucleic acid and, 305
cultured cells and, 606
in deoxyribonucleic acid, 642-49
- 5-Methyldeoxycytidine non-random incorporation of, 178
- 3-O-Methyl-D-glucitol periodate oxidation of, 47
- Methyl- β -D-glucopyranoside oxidation of, 49
- Methylglyoxal biosynthesis of, 252
- 1-Methyl histidine vitamin E and excretion of, 462
- 4-Methyl-5-(β -hydroxyethyl)thiazole thiamine biosynthesis and, 430
- 6-Methyl-7-hydroxy-8-ribityl lumazine isolation of, 418
- Methylmalonyl coenzyme A biotin and metabolism of, 388
isomerization of, 201
vitamin B₁₂ and isomerization of, 439
- Methylmalonyl isomerase vitamin B₁₂ and, 201-2
- Methylphosphates biochemistry of, 255
- Methylpurine acceptor ribonucleic acid

- and, 305
- riboflavin biosynthesis and, 411
- 2-0-Methylribose
 - acceptor ribonucleic acid and, 305
- Methylthioadenosine
 - formation of, 585-86
- 1-Methylthymine
 - crystal structure of, 115
- Methyltryptophan
 - tryptophan utilization and, 302
- 5-Methyluridine
 - synthesis of, 138
- Methyluridylic acid
 - polymerization of, 151
- Metmyoglobin
 - kinetics of activity, 31-32
- Mevaldic acid
 - mevalonic acid biosynthesis and, 526
- Mevalonic acid
 - carotene biosynthesis and, 541
 - cholesterol biosynthesis and, 527-33, 535
 - coenzyme Q biosynthesis and, 542
 - digitoxigenin biosynthesis and, 544
 - ergot alkaloid biosynthesis and, 544
 - rubber biosynthesis and, 543
 - synthesis of, 525-27
- Mevalonic acid kinase
 - purification of, 527
- Mevalonic acid-5-phosphate
 - metabolism of, 527-28
- Mevalonic acid-5-pyrophosphate
 - metabolism of, 528
- Mice
 - protein nutrition and, 330
- Microorganisma
 - cytochrome c in, 567-69
 - lipids of, 86-87
 - nucleic acids of, 316
 - polyamines in, 581-82
 - protein assay by, 334-35
 - spermine effect on, 598
- Microscopy
 - fat absorption and, 189
- Microsomes
 - isotope transfer to, 169
 - protein synthesis and, 308-14
 - relaxing factor and, 488
 - spermine agglutination of, 600-1
- Milk
 - protein nutrition and, 326
- Mitochondria
 - cytochrome b_5 and, 16
 - dehydrogenases of, 34
 - isolation of, 28
 - mitosis and, 678
 - permeability of, 25
 - polyamines, 593
 - potassium transport in, 474
 - protein synthesis in, 308
 - spermine agglutination of, 600-1
 - swelling of, 26
- Mitochrome
 - oxidative phosphorylation uncoupling by, 23
- Mitosis
 - apparatus of, 674-77
 - cultured cells and, 617
 - energetics of, 677-79
 - glutathione and, 681
 - mitochondria and, 678
 - respiration and, 677-78
 - sulfate and, 681-82
- Mitotic apparatus
 - chemistry of, 675-76
 - components of, 672
 - enzymes of, 676
 - intermolecular bonding and, 676-77
 - isolation of, 675
- Mitotic division
 - ionizing radiation and, 182-83
- Monoacetylspermine
 - in microorganisms, 582
- Monoamine oxidase
 - mechanism of, 245
- Monoglycerides
 - phosphorylation of, 196-97
- Monomethylethanolamine
 - existence of, 81
- Monophosphoinositide
 - structure of, 391-92
- Moulds
 - lipids of, 86-87
- Mucopolysaccharide
 - cell culture and, 610
- Muscarine
 - x-ray diffraction of, 126
- Muscle
 - anserine in, 490
 - biochemistry of, 473-98
 - carbohydrate transport in, 221-22
 - carnosine in, 490
 - contractile system in, 474-86
 - contraction of, 486-87
 - kwashiorkor and changes in, 340
 - minor components of, 485-86
 - non-contractile system in, 473-74
 - non-protein nitrogen of, 489-91
 - nucleic acids in, 491-92
 - phosphagen in, 489-90
 - protein sparing action in, 327
 - protein structure of, 101-2
- relaxing factor, 487-89
- Mutagenesis
 - biochemistry of, 660-63
 - chemical agents for, 660-61
- Mutation
 - cell culture and, 626-29
 - deoxyribonucleic acid and, 294
 - deoxyribonucleic acid structure and, 651
 - protein levels and, 654
- Mycellianamide
 - biosynthesis of, 543
- Mycobacteria
 - lipids of, 87
- Mycophenolic acid
 - biosynthesis of, 543
- Mycosides
 - chemistry of, 85
- Myeloperoxidase
 - porphyrin of, 30
- Myofibrils
 - localization of components in, 47
 - minor components of, 485-86
- Myogen
 - electrophoresis of, 473
- Myoglobin
 - biochemistry of, 557-64
 - conformation of, 270-73
 - deuterium exchange in, 270-71
 - isomorphous replacement and, 104
 - optical rotatory dispersion of, 270
 - physicochemical studies on, 558-60
 - structure of, 105-10
- Myosin
 - adenosine triphosphatase activity of, 480-83
 - chromosome movement and, 683
 - localization of, 474
 - nucleic acid and, 491
 - size of, 477
 - spectroscopy of, 285
 - subunits of, 477

N

- Nebularine
 - degradation of, 138
- Negative feedback
 - in mammals, 245-46
- Neisseria perflava
 - polyamines and, 591
- Nemotinic acid
 - structure of, 76
- Nerolidol pyrophosphate
 - metabolism of, 530-31
- Neuramidase
 - biochemistry of, 375
- Neuraminic acid

- in gangliosides, 84
 - Niacin
 - see Nicotinic acid
 - Niacytin
 - structure of, 394-95
 - Nicotinamide
 - determination of, 397
 - infant metabolism and, 329
 - metabolism of, 396-97
 - nicotinamide adenine di-nucleotidase inhibition by, 25
 - Nicotinamide adenine di-nucleotidase
 - inhibition of, 25
 - Nicotinamide adenine di-nucleotide
 - ascorbic acid and, 16-17
 - biosynthesis of, 397
 - ketose reductase and, 229
 - low temperature reduction of, 26
 - mitochondrial permeability and, 25
 - precursor release, 25-26
 - vitamin K reductase and, 23
 - Nicotinamide adenine di-nucleotide H₂
 - deoxyriboside synthesis and, 175
 - Nicotinamide adenine di-nucleotide H₂ oxidase
 - steroid effect on, 517
 - Nicotinamide adenine di-nucleotide phosphate
 - aldose reductases and, 229
 - mevalonate biosynthesis and, 525-26
 - Nicotinamide adenine di-nucleotide phosphate H₂
 - fatty acid synthesis and, 194
 - steroid metabolism and, 502
 - Nicotinic acid
 - biochemistry of, 394-97
 - biosynthesis of, 394
 - biotin deficiency and, 389-90
 - cholesterol synthesis and, 540
 - metabolic effects of, 395-96
 - metabolism of, 396-97
 - nutrition and, 394-95
 - pyridine nucleotide synthesis and, 397
 - Nicotinuric acid
 - determination of, 397
 - excretion of, 396
 - Night blindness
 - vitamin A and, 450-51
 - Nitrate
 - assimilation of, 239-40
 - Nitrite
 - reduction of, 240
 - Nitrogen
 - metabolism of, 239-41
 - Nitrosobenzene
 - hemoglobin binding of, 559
 - Nitrous acid
 - mutagenesis and, 661
 - Nocardic acid
 - structure of, 78
 - Norepinephrine
 - metabolism of, 257-58
 - Norleucine
 - protein nutrition and, 330
 - protein synthesis and, 302
 - Noviose
 - synthesis of, 56
 - Nuclear magnetic resonance
 - of lipids, 74
 - Nucleic acid
 - biosynthesis of, 165-88
 - chemical and physical properties of, 641-50
 - chemistry of, 133-64
 - cultured cells and, 617-18
 - dye binding by, 155-56
 - fractionation of, 140
 - metabolism of, 616-18
 - in muscle, 490-91
 - nucleotide sequence, 139-41, 641-43
 - polyamine binding by, 595
 - radiation and synthesis of, 631
 - riboflavin biosynthesis and, 411
 - terminal nucleotides in, 641-42
 - transforming principle, 658-59
 - viral infection and, 658-59
 - vitamin E and levels of, 461
 - x-ray diffraction of, 117-19
 - Nucleolus
 - chromosome coiling and, 674
 - ribonucleic acid synthesis in, 171, 173
 - Nucleoprotein
 - irradiation of, 184
 - in muscle, 486
 - nucleic acid organization in, 158
 - x-ray diffraction of, 119-21
 - Nucleoside-2',3'-cyclic phosphate
 - polymerization of, 151-53
 - Nucleoside diphosphates
 - ribonucleic acid synthesis and, 165
 - synthesis of, 139, 143
 - Nucleosides
 - chemistry of, 133-38
 - oxidation of, 138
 - phosphorylation of, 139
 - riboflavin biosynthesis
 - and, 411
 - Nucleoside triphosphate
 - irradiation of, 181-82
 - non-terminal incorporation of, 170-71
 - synthesis of, 143-45
 - terminal incorporation of, 167-70
 - Nucleotide
 - acceptor ribonucleic acid and, 304-5
 - chemistry of, 138-39
 - deoxyribonucleic acid and sequence of, 295
 - in muscle, 490-91
 - ribosomal ribonucleic acid and, 310
 - sequence determination, 641-43
 - unusual structure, 305
 - uptake in cultured cells and, 622
 - in vitamin B₁₂, 436-37
 - x-ray diffraction of, 114-15
 - Nucleotide anhydrides
 - chemistry of, 141-46
 - Nucleotide coenzymes
 - synthesis of, 142-43
 - Nucleotide kinases
 - phage infection and, 316
 - Nucleotide transosylases
 - biochemistry of, 359-64
 - Nucleus
 - protein synthesis in, 308
 - ribonucleic acid synthesis in, 170-71
 - Nutrition
 - proteins and, 325-46
- O
- Oils
 - fatty acid content of, 75
 - Oligonucleotides
 - dye binding by, 155-56
 - mercuric chloride interaction with, 156
 - pK values of, 156
 - separation of, 642
 - synthesis of, 147
 - Oligosaccharides
 - uridine diphosphate glucose and synthesis of, 359-60
 - Opsopyrroledicarboxylic acid
 - porphyrin biosynthesis and, 552
 - Optical rotation
 - of carbohydrates, 45-46
 - Optical rotatory dispersion
 - of myoglobin, 270
 - of ribonuclease, 282
 - Ornithine
 - cell culture and, 607

nicotinic acid conjugate
of, 396
Ornithine transcarbamylase
biotin deficiency and, 390
Ovary
biosynthesis of hormones,
503-7
Oxalacetate
biotin and synthesis of,
389
cell culture and, 609
Oxalate
biosynthesis of, 251
relaxing factor and, 488-
89
Oxalate calculi
pyridoxine and, 403-4
Oxidases
amino acid metabolism
and, 245
Oxidation
of aromatic compounds,
258-59
Oxidation, biological
amino acid oxidase, 37
catalase, 32-34
cytochrome a, 11-15
cytochrome b, 18-20
cytochrome b₂, 17-18
cytochrome b₅, 16-17
cytochrome c, 15-16
cytochrome c₁, 20
glutamic dehydrogenase,
34-35
 α -ketoglutarate, 35-36
lipids, 20-24
oxidative phosphoryla-
tion, 26-30
peroxidase, 30-32
pyridine nucleotides, 24-
26
recent developments in,
11-44
succinic dehydrogenase,
24
xanthine oxidase, 36-37
Oxidative phosphorylation
inhibitors and activators
of, 23
in vivo studies of, 27
model system for, 146
recent developments in,
26-30
vitamin K and, 464-65
2-Oxo-L-gulonic acid
configuration of, 46
Oxygen
biological reduction of,
14-15
cell culture and, 611
Ozonolysis
of fatty acids, 79

P

Palmityl coenzyme A
oxidation of, 196

Palustrin
spermidine in, 583-84
Panose
biosynthesis of, 355
Paramagnetic resonance
cytochrome-c-oxidase
and, 13
Parathyroid hormone
vitamin A and, 453
vitamin D and, 456
Parinic acids
stereochemistry of, 75,
79
Pasturella tularensis
polyamines and, 591
Pathology
and polyamines, 597-600
Pea flour
protein nutrition and, 337
Peanuts
protein nutrition and, 336
Pellagra
leucine intake and, 394
Pentenoic acid derivatives
cholesterol synthesis
inhibition by, 536
Pentosans
biosynthesis of, 361-62
Pentose phosphate pathway
recent developments of,
232-33
Pepsin
ribonuclease digestion by,
281
spectroscopy of, 286
Peptidase
mitosis and activity of,
680
Peptide bond
planarity of, 96
Peptides
lipid and, 85
penetration of, 241
uridine-5-pyrophosphate
binding of, 142
x-ray diffraction of, 95-97
Peptidolipids
chemistry of, 85-86
Perch scales
nutritional value of, 335-
36
Periodate oxidation
of carbohydrates, 46-48
of ribonucleic acid, 301
Permeases
carbohydrate metabolism
and, 213-18
Peroxidase
biological oxidation and,
30-32
hemin combination and,
563
inhibition of, 33
mechanism of, 32
Peroxides
vitamin E levels and, 21
Pharmacology
of polyamines, 597-600
Phenylalanine
amino acid imbalance and,
332
crystal structure of, 95
lipid and, 85
metabolism of, 256-59
protein nutrition and, 330
Phenylidosoacetate
glycol cleavage by, 47
Phenylketonuria
amine metabolism and,
259
Phloretin
sugar transport inhibition
by, 218
Phlorizin
intestinal absorption in-
hibition by, 220
sucrose transport and,
217-18
Phosphagens
in muscle, 489-90
Phosphatase
mitotic apparatus and,
676
mutations and, 294
polyamines and, 595
vitamin D and activation
of, 457
Phosphate
actin polymerization and,
476
actomyosin binding of,
484
muscle contraction and,
486-87
vitamin D and deposition
of, 456-57
vitamin E and turnover of,
461
Phosphatides
chromatography of, 73
Phosphatidic acid
biosynthesis of, 196-97
existence of, 81
Phosphatidyl inositol
structure of, 392
3'-Phosphoadenosine-5'-
phosphosulfate
vitamin A and, 453
Phosphoethanolamine
biosynthesis of, 251
Phosphoglucomutase
properties of, 226
Phosphoglucose isomerase
properties of, 227
Phosphoglyceric acid mutase
varieties of, 228
Phosphohexose isomerases
inhibition of, 226-27
O-Phosphohomoserine
metabolism of, 252
Phospholipase A
site of action, 198
Phospholipid
biosynthesis, 196-99

- chromatography of, 73
 natural occurrence of, 81-83
 synthesis of, 79-81
 Phosphomevalonate kinase
 purification of, 528
 Phosphoramidates
 phosphorylation with, 143
 Phosphorochloridates
 polynucleotide synthesis and, 146-47
 Phosphorylase
 activation of, 225-26
 pyridoxal phosphate content of, 364
 vitamin E and levels of, 461
 Phosphorylase a
 pyridoxal phosphate content of, 399-400
 Phosphorylase b
 characterization of, 400
 Phosphorylase c
 characterization of, 400
 Phosphoserine
 metabolism of, 250-51
 Phosphotransosylases
 biochemistry of, 348-49
 Photosynthesis
 ascorbic acid function and, 385
 Phytic acid
 zinc and, 393
 Pinene
 biosynthesis of, 543
 Pinocytosis
 triglyceride absorption and, 189-92
 Pipelic acid
 lysine metabolism and, 253
 Pituitary hormones
 fatty acid metabolism and, 203-4
 lipid metabolism and, 204
 Placenta
 biosynthesis of hormones in, 508
 sugar transport in, 221
 Plantose
 synthesis of, 357
 Plasmalogen
 metabolism of, 199
 structure of, 82
 Plasmaïnogen
 nicotinic acid activation of, 396
 Plastoquinone
 relation to coenzyme Q, 542
 Pleuropneumonia organisms,
 cell culture and, 631
 Pneumococcal polysaccharide type III
 biosynthesis of, 363
 Polio virus
 cultured cells and, 629
 structure of, 124
 Polyadenylic acid
 biosynthesis of, 166
 oligouridylic acid interaction with, 157
 polyamine binding by, 596
 Poly-L-alanine
 helical structure of, 97-98
 Polyamines
 biochemistry of, 579-604
 ribosome integrity and, 308
 see also Spermine
 Polycytidylic acid
 pK values of, 151
 Polyglycine
 structure of, 98-99
 Polyguanylic acid
 pK values of, 156
 streptolysin S induction by, 150
 synthesis of, 166
 Polyhydric alcohols
 benzaldehyde reaction with, 62
 Polyinosinic acid
 x-ray diffraction of, 118
 Polynucleotide
 chemical synthesis of, 650
 interactions of, 157
 physical chemistry of, 158-59
 physical properties of, 153-58
 stepwise degradation of, 50
 synthesis of, 146-53
 x-ray diffraction of, 117-19
 Polynucleotide phosphorylase
 biochemistry of, 165-67
 mechanism of action, 166-67
 Polypeptides
 x-ray diffraction of, 97-98
 Polyploidy
 cultured cells and, 627-28
 Poly-L-proline
 structure of, 98
 Polyriboadenylic acid
 x-ray diffraction of, 118
 Polysaccharides
 desulfation of, 63
 uridine diphosphate sugars and, 361-64
 Polyuridylic acid
 oligoadenylic acid interaction with, 157
 pK values of, 150
 polyamine binding by, 596
 ultraviolet irradiation of, 157
 x-ray diffraction of, 118
 Polyvinylpyrrolidine
 cell culture and, 610
 Porphobilinogen
 biosynthesis of, 550-51
 Porphobilinogen aldehyde
 porphyrin synthesis and, 551
 Porphobilinogenase
 action of, 554
 Porphobilinogen deaminase
 action of, 554
 Porphyrin
 biosynthesis of, 549-57
 α -amino levulinic acid and, 550
 coproporphyrinogen and, 555-56
 iron and, 556-57
 porphobilinogen and, 550-51
 protoporphyrin and, 556
 uroporphyrinogen and, 551-55
 chemical synthesis of, 553
 myeloperoxidase and, 30
 vitamin B₁₂ biosynthesis and, 435-36
 Prednisolone
 see Steroid hormones
 Prednisone
 see Steroid hormones
 Prefolic acid A
 characterization of, 425-26
 Pregnancy
 pyridoxine deficiency and, 403
 Pregnanedione
 see Steroid hormones
 Pregnanetriol
 see Steroid hormones
 Pregnenolone
 see Steroid hormones
 Prephenic acid
 metabolism of, 256
 Primer
 deoxyribonucleic acid polymerase and, 177
 polyguanylic acid and, 166
 Prodigiosin
 porphyrin biosynthesis and, 555
 Proflavine
 polyamines and, 592
 Progesterone
 biological oxidation and effect on, 22
 cell culture and, 611
 effects of, 515
 vitamin A and, 451-52
 see also Steroid hormones
 Prolactin
 lipid metabolism and, 204
 Proline
 collagen and, 99
 crystal structure of, 95
 cultured cells and, 614

metabolism of, 254
 Propamide
 polyamines and, 592
 Propionic acid
 metabolism of, 201-3
 Propionyl coenzyme A
 biotin and metabolism of, 388
 Propylamine transferase
 action of, 586
 Protamine
 cholesterol synthesis and, 538-39
 Protease
 mitosis and activity of, 680
 Protein
 cell content of, 606
 cell culture and, 609-11
 heterogeneity of, 295
 metabolism of
 cultured cells and, 614-15
 nutrition and, 332-33
 mitotic apparatus and, 672, 675-76
 nutrition and, 325-46
 amino acid deficiency and, 331
 amino acid imbalance, 331-32
 amino acid requirements and, 327-31
 foodstuffs and diets, 33-37
 kwashiorkor and, 337-41
 metabolism and, 332-33
 protein requirements, 326-27
 in ribosomes, 309
 structure of, 269-92
 hemoglobin, 270-77
 hydrophobic bonds and, 288
 mutations and, 652-53
 myoglobin, 270-77
 ribonuclease, 277-84
 ultraviolet spectral shifts and, 284-89
 synthesis of
 additional ribonucleic acid and, 314-17
 base analogues and, 314
 deoxyribonucleic acid and amino acid sequence, 293-98
 enucleate cells and, 296
 heterologous ribonucleic acids, 312-13
 other systems of, 317
 pH 5 precipitation and, 312-13
 ribonucleic acid and amino acid sequence, 298-317
 ribosomes and, 308-14
 specificity in, 293-324

vitamin B₁₂ and, 439
 turnover of, 620
 x-ray diffraction of, 97-113
 Protoplasts
 polyamine stabilization of, 593
 Protoporphyrin
 biosynthesis of, 556
 Pseudoglobulin
 myofibril content of, 485
 Pseudoporphyrin
 porphyrin biosynthesis and, 555
 Pseudovitamin B₁₂
 structure of, 437-38
 Pteridines
 biosynthesis of, 409-27
 Pteroylglutamate
 interconversion of derivatives of, 425-26
 metabolism of, 421
 Purines
 biosynthesis of, 409-27
 cultured cells and, 616
 irradiation of, 184
 x-ray diffraction of, 115-16
 Putresine
 relation to spermine, 579
 Pyranose ring
 hydrogen bond in, 58-59
 Pyridine nucleotides
 biological oxidation and, 24-26
 biosynthesis of, 397
 folic acid metabolism and, 426
 glutamic dehydrogenase and, 34-35
 oxidative phosphorylation and, 27
 stereospecificity of enzymes utilizing, 35
 Pyridoxal
 cell culture and, 608
 cysteine reaction with, 404-5
 metabolism of, 401-2
 see also Vitamin B₆
 bis-4-Pyridoxal disulfide formation of, 404-5
 Pyridoxal phosphate
 adenosine triphosphatase inhibition and, 488-89
 amino acid metabolism and, 242-43
 porphyrin biosynthesis and, 556
 Pyridoxamine
 metabolism of, 401-2
 see also Vitamin B₆
 Pyridoxic acid
 metabolism of, 401-2
 Pyridoxine
 amino acid absorption and, 403

metabolism of, 401-2
 plasma lipid levels and, 403
 pregnancy and deficiency of, 403
 Pyrimethamine
 folic acid metabolism and, 427
 Pyrimidine
 biosynthesis of, 616
 crystal structure and, 115
 irradiation of, 184
 riboflavin biosynthesis and, 411-12
 thiamine biosynthesis and, 427
 x-ray diffraction of, 115-16
 Pyrophosphatase
 ribonucleic acid synthesis and, 167
 Pyrophosphate
 actomyosin dissociation, 479
 crystal structure of, 115
 Pyrophosphorylation
 of acceptor ribonucleic acid, 305
 Pyruvic acid
 carboxylation of, 389
 cell culture and, 609
 decarboxylation of, 433-34
 kwashiorkor and, 339
 propionic acid synthesis and, 201
 reactions of, 233

Q

Queen substance
 structure of, 76
 Quercetin
 galacturonide biosynthesis, 360
 Quinacrine
 polyamines and, 592
 Quinaldic acid
 biosynthesis of, 261
 Quinine
 polyamines and, 592
 Quinolinic acid
 nicotinic acid synthesis and, 394

R

Rachitis
 vitamin D and, 456
 Radiation
 cell culture and, 631
 deoxyribonucleic acid and, 661-63
 Raffinose
 levansucrase action on, 352
 Rats
 protein nutrition and, 330
 Refractive index

- protein spectroscopy and, 287-88
- Relaxing factor
action of, 487-89
- Renal calculi
pyridoxine deficiency and, 403-4
- Respiration
mitosis and, 677-78
- Reticulocytes
hemoglobin synthesis in, 312
- Retinene
formation of, 451
- L-Rhamnose
thymine nucleotides and synthesis of, 363-64
- Rhodopsin
reactions of, 450-51
- Riboflavin
biochemistry of, 409-27
inhibition of synthesis of, 412
lactic dehydrogenase and, 18
- Ribonuclease
amino acid sequence in, 277
antigenicity of, 281
cholesterol synthesis and, 338
deuterium exchange in, 281-82
heterogeneity of, 278
physical chemistry of, 281-84
reoxidation of sulphydryl groups of, 295
spectroscopy of, 284-85
spermine inhibition of, 600
structural requirements for activity, 278-81
structure of, 102
tertiary structure of, 277
titration of, 283
- Ribonuclease A
regeneration of, 280
- Ribonuclease digest
aminoacyl adenosine in, 301
oligonucleotides in, 642
- Ribonucleic acid
acceptor
base composition of, 304-5
fractionation of, 307
helical configuration of, 304
hemoglobin synthesis and, 312
heterogeneity of, 306-7
hydrogen bonding in, 170
polynucleotide phosphorylase and, 167
pyrophosphorolysis of, 305
- ribosomal binding of, 310-11
size of, 303
specificity of, 303
spermine fractionation of, 596
'taka'-diastase hydrolysis of, 306
terminal groups of, 169
terminal nucleotide sequence in, 167-68
turnover of, 315
amino acid sequence and, 298-317
amino acid specificity of, 301
amino acyl derivatives of, 300
biosynthesis of, 165-74
cell content of, 606
in centriole, 672
chloroplast synthesis of, 172
in chromosomes, 674
cultured cells and, 620-21
deoxyribonucleic acid hybrid with, 316
deoxyribonucleic acid hydrogen bonding to, 171
hydrogen bonding in, 649-50
instability of, 649
mitotic apparatus and, 675
nucleotide sequence of, 641-43
physical properties of, 649
preparation of, 649
protein synthesis and, 314-17
ribosomal
hydrogen bonding in, 309-10
nucleotide composition of, 310
size of, 309
turnover of, 315
secondary structure of, 158
sites of synthesis, 171-74
spermine fractionation of, 596
synthesis
cell division and, 673
cultured cells and, 616-17
mitosis and, 650
phage infection and, 315-16
role of deoxyribonucleic acid in, 171
x-ray diffraction of, 118-19
- Ribonucleoprotein
cholesterol synthesis and, 538
- Ribonucleotides
polymerization of, 149-50
- Ribose
cultured cells and, 616
transport of, 216
- Ribose-5-phosphate
histidine biosynthesis and, 253
- Ribosides
reduction to deoxyribosides, 175
- α -Ribosides
vitamin B₁₂ and, 437
- Ribosomes
acceptor ribonucleic acid binding to, 311
amino acid sequence and, 310-14
instability of, 315
polyamines in, 582-83
protein synthesis and, 308-14
stabilization of, 594
structure of, 308-10
x-ray diffraction of, 119-20
- 5-Ribosyluracil
distribution of, 169
- 5-Ribosyluridylic acid
acceptor ribonucleic acid and, 305
polymerization of, 150
ribosomal ribonucleic acid and, 310
- Ribothymidylic acid
acceptor ribonucleic acid and, 305
- Ricinoleic acid
structure of, 75
- Rosaniline
oligonucleotide binding of, 155-56
- Rosenonolactone
biosynthesis of, 543
- Rubber
biosynthesis of, 543
- S
- Salmine
cell culture and, 610
- Salts
cell culture and, 609
- Sarcinic acid
structure of, 76
- Sarcoplasm
granular components in, 474
muscle biochemistry and, 473
- Sarcoplasmic reticulum
nature of, 488
- Sarcosomes
oxidative phosphorylation in, 474
- Scar tissue
ascorbic acid levels

- and, 385
 Schardinger dextrins
 synthesis of, 351-52
 Sedimentation
 of ribosomes, 308-9
 Sedimentation coefficient
 of deoxyribonucleic acids,
 644-45
 Selenium
 liver necrosis and, 20-21
 Selenomethionine
 protein synthesis and, 302
 Semen
 polyamines in, 580
 Serine
 analysis for, 75
 cell culture and, 606
 crystal structure of, 95
 metabolism of, 250-51,
 613
 riboflavin synthesis and,
 410
 D-Serine
 metabolism of, 251
 Serotonin
 metabolism of, 260
 phosphorylase activation
 by, 226
 Serum
 cell culture and, 609-10
 cholesterol levels in, 395
 proteolytic enzymes in,
 611
 Serum protein
 kwashiorkor and, 3
 Sialic acid
 neuraminidase and, 375
 Silk
 structure of, 98
 Sodium borohydride
 selective reduction by,
 47
 Solanesol
 relation to coenzyme Q,
 542
 Somatotrophin
 cell culture and, 611
 Sorbitol
 foetal fructose and, 221
 Sorbitol dehydrogenase
 action of, 229-30
 Soyasapogenol
 biosynthesis of, 543
 Soybean
 protein nutrition and, 336
 Spectroscopy
 of deoxyribonucleic acid,
 647
 of proteins, 284-89
 Spermidine
 see Spermine
 Spermine and spermidine
 assay techniques, 579-80
 biochemistry of, 579-604
 biosynthesis of, 584-86
 chemical synthesis of, 584
 distribution and concentra-
 tion of, 580-84
 heparin binding by, 597
 lipid binding by, 596-97
 metabolism of, 586-90
 microbiological require-
 ments for, 590-92
 miscellaneous effects of,
 600-1
 nucleic acid binding by,
 595-96
 pharmacology of, 597-600
 stabilizing effects of, 592-
 95
 Spermine oxidase
 action of, 587-88
 Shingomyelin
 chemistry of, 83
 chromatography of, 73
 synthesis of, 81
 Sphingosine
 chromatography of, 73
 Spindle
 chromosome movement
 and, 682
 synthesis of, 672
 Spleen
 albumin breakdown in,
 333
 Spongothymidine
 synthesis of, 136
 Spongouridine
 synthesis of, 136
 S Protein
 protein synthesis and,
 317
 Squalene
 biosynthesis of, 527-33
 cholesterol biosynthesis
 and, 533-35
 Staphylococcus aureus
 polyamines in, 582
 spermine inhibition of,
 598
 Steroid hormones
 adrenal, 500-2
 androgen biosynthesis, 507
 androgen effects, 515-16
 biochemistry of, 499-524
 biosynthesis of, 500-8
 biosynthesis of C₂₁ ster-
 oids-table, 502
 catabolism of C₂₁ ster-
 oids-table, 510
 corticosteroid effects,
 514-15
 degradation of, 207-8
 dehydroepiandrosterone,
 502-3
 effects of, 514-18
 enzymes affected by, 517-
 18
 estrogen effects, 516-17
 6-hydroxylation of, 512
 7-hydroxylation of, 513
 16 α -hydroxylation of, 513
 18-hydroxylation of, 513
 hydroxysteroid dehydro-
 genases, 509-12
 in vitro biosynthesis-
 table, 504-5
 isolated steroids-table,
 506-7
 Δ^5 -3-keto isomerization
 of, 514
 ketosteroid reductases,
 509-12
 ovary and, 503-7
 progesterone effects, 515
 Δ^4 -reductases, 508-12
 side chain cleavage, 514
 sulfate ester synthesis of,
 514
 systematic nomenclature
 for, 499
 testis, 507-8
 transformation of, 508-14
 Steroid metabolism
 cultured cells and, 618
 Sterol
 nicotinic acid and metabo-
 lism of, 395
 Streptolysin S
 polyguanylic acid induction
 of, 150
 Subtilisin
 ribonuclease modification
 by, 279
 Succinate
 cytochrome b reduction
 by, 18
 Succinic dehydrogenase
 biological oxidation and,
 24
 vitamin E and activity of,
 461
 Succinoxidase
 free radicals formed by,
 24
 Succinyl coenzyme A
 porphyrin synthesis and,
 550
 Sucrose
 cell culture and, 609
 chemical oxidation of, 47
 dextran synthesis from,
 349-50
 levan biosynthesis from,
 352-53
 mechanism of hydrolysis
 of, 366
 partial methylation of, 65
 synthesis of amylopectin
 from, 350
 transport of, 217
 uridine diphosphate
 glucose and synthesis
 of, 359-60
 Sucrose-6-phosphate
 uridine diphosphate glu-
 cose and synthesis of,
 359-60
 Sucrose phosphorylase
 biochemistry of, 348
 donor specificity of, 367

- equilibrium constant, 365
 Sugar nucleotides
 transosylases and, 359-64
 Sugars
 guanosine diphosphate derivatives of, 142
 thymidine diphosphate derivatives of, 142
 transport of, 213-23
 Sulfates
 of carbohydrates, 63-64
 cell division and, 681
 glycogen synthetase activation by, 225
 vitamin A and, 453
 Sulfhydryl groups
 in hemoglobin, 276, 558
 reoxidation of, 295-96
 transosylation active site and, 370
 Sulfite
 reduction of, 240
- T
- Taka-amylase
 carbohydrate content of, 364
 Taka-diastase
 ribonucleic acids hydrolysis by, 306
 Taurine
 absorption of, 241
 cultured cells and, 621
 Terpenoid acids
 biosynthesis of, 531-32
 Testis
 biosynthesis of hormones in, 507-8
 Testosterone
 see Steroid hormones
 Tetrahydroaldosterone
 see Steroid hormones
 Tetrahydrocortisol
 see Steroid hormones
 Tetrahydrocortisone
 see Steroid hormones
 Tetrahydrofolic acid
 biosynthesis of, 425
 isomerization of N-formyl derivatives of, 426-27
 Tetrahydropyran
 hydrogen bonding of derivatives of, 58
 1, 3, 7, 9, -Tetramethyluric acid
 crystal structure of, 115
 Tetrapyrrolylmethane structure
 porphyrin synthesis and, 552
 Tetrazolium reductase
 ubiquinone and, 22
 Theophylline
 crystal structure, 115
 hydrogen bonds in, 115
- Thiamine
 biochemistry of, 427-35
 derivatives of, 433
 Thiamine monophosphate
 metabolism of, 429
 Thiamine pyrophosphate
 biosynthesis of, 428, 433
 Thiation
 of purines and pyrimidines, 137
 Thiazole
 analogues of, 430-31
 biosynthesis of, 431-32
 α -glycerophosphate dehydrogenase and, 435
 thiamine biosynthesis and, 427
 Thiazolidine
 biosynthesis of, 432
 β -Thienylalanine
 cultured cells and, 615
 Thioglycosidases
 specificity of, 366
 Thiols
 mitosis and, 680-82
 2-Thiouridine-5'-diphosphate
 polynucleotide phosphorylase and, 165-66
 5'-Thiouridylic acid
 polymerization of, 151
 Threonine
 amino acid imbalance and, 332
 crystal structure of, 95
 cultured cells and, 613
 metabolism of, 252
 protein nutrition and, 330
 riboflavin biosynthesis and, 410-11
 valyl-ribonucleic acid synthetase and, 301-2
 Threonyl-ribonucleic acids
 formation of, 300
 Threose-diphosphate
 inhibitory action of, 227-28
 Thymidine
 cell culture and, 608
 synthesis of, 133
 Thymidine diphosphate
 sugar derivatives of, 142, 363
 Thymidine kinase
 x-ray irradiation and, 183
 Thymidylic acid
 crystal structure of, 115
 polymerization of, 147
 Thymidyl-3':5'-thymidine
 synthesis of, 146-47
 Thymine
 irradiation of, 157, 662
 vitamin B₁₂ and synthesis of, 440-41
 Thymus
 deoxyribonucleic acid polymerase and, 179
 Thyroid hormones
 ascorbic acid function and, 387
 lipid metabolism and, 205
 Thyroxine
 cultured cells and, 619
 oxidative phosphorylation and, 29
 vitamin A and, 452
 vitamin B₆ and, 404-5
 Tissue culture
 nutrition and, 331
 Tobacco mosaic virus
 amino acid alterations and, 654
 amino acid sequence in, 123
 irradiation of, 184
 protein synthesis and, 298-99
 renaturation of proteins in, 295
 ribonucleic acid structure in, 158
 spectroscopy of protein from, 284-85
 structure of, 121-23
 x-ray irradiation of, 662
 Tocols
 chemistry of, 458
 Tocopherol
 antioxidant activities of, 462
 α -Tocopherol
 electron transport and, 459
 α -Tocopherol
 structure of, 458
 ϵ -Tocopherol
 structure of, 458
 Tocopherols
 see Vitamin E
 Tocopherylquinones
 presence of, 460
 Toluene-p-sulfonyl chloride
 polynucleotide synthesis and, 147
 Toluidine blue
 oligonucleotide binding by, 155-56
 Transaldolase
 action of, 232
 Transaminase
 characterization of, 398
 cultured cells and, 612
 pyridoxal binding by, 243
 serum levels of, 402-3
 Transamination
 of alanine, 248-49
 of amino acids, 242-43
 mechanism of, 398
 Transcarnbamylase
 requirements of, 248
 Transcarboxylations
 biotin and, 389
 Trans-N-deoxyribosidase
 action of, 177
 Transferrin
 ascorbic acid function

and, 385-86
 Transforming principle
 nucleic acids and, 658-59
 Transfructosylases
 biochemistry of, 352-53
 transosylases-glycosidases, 353-54
 Trans- α -galactosylases
 biochemistry of, 358-59
 Trans- β -galactosylases
 biochemistry of, 357-58
 Transglucosylases
 true transosylases and, 349-52
 Trans- α -glucosylases
 biochemistry of, 354-56
 Trans- β -glucosylases
 biochemistry of, 356-57
 Trans- β -glucuronosylases
 biochemistry of, 359
 Transglutaminase
 polyamines and, 590
 Transglycosidation
 biochemistry of, 347-82
 Trans-O-glycosylases
 configuration of products, 367
 Transketolase
 action of, 232
 Transosylases
 acceptor specificity, 368
 donor specificity, 367-68
 Transosylases-glycosidases
 biochemistry of, 353-59
 Transosylation
 definition of, 347-48
 enzyme properties, 364-75
 mechanism of, 368-71
 Transport system
 for carbohydrates, 213-14
 Trehalase
 characterization of, 355
 Trehalose-phosphate
 uridine diphosphate glucose and synthesis of, 360
 Tricarboxylic acid cycle
 porphyrin synthesis and, 550
 vitamin D and, 457
 Tricothecin
 biosynthesis of, 543
 Trideoxynucleoside diphosphate
 synthesis of, 149
 Triglyceride
 absorption of, 189-90
 biosynthesis of, 196-99
 epinephrine effect on, 204-5
 metabolism of, 190-92
 Trimethylsilyl ethers
 gas chromatography of, 45
 Triosephosphate isomerase
 mechanism of, 227
 Triparanol
 inhibition of cholesterol synthesis by, 536

Triphosphate
 crystal structure of, 115
 Triphosphoinositide
 structure of, 391-92
 Triphosphopyridine nucleotide
 see Nicotinamide adenine dinucleotide phosphate
 Tripyrrylmethane structures
 porphyrin biosynthesis and, 552
 Tropomyosins
 characterization of, 484-85
 localization of, 474-75
 True transosylases
 biochemistry of, 349-53
 Trypsin
 cultured cells and, 619
 myosin and, 478
 Trypsinogen
 spectroscopy of, 285
 Tryptophan
 abnormal metabolism of, 404
 analogues of, 302
 ascorbigen structure and, 385
 crystal structure of, 95
 ergot alkaloid biosynthesis and, 544
 metabolism of, 259-61
 myoglobin and location of, 107
 negative feedback and, 247
 nicotinic acid synthesis and, 394
 protein nutrition and, 328
 protein spectroscopy and, 286
 Tryptophanase
 action of, 259
 Tryptophan peroxidase
 induction of, 30
 Tryptophan pyrrolase
 induction of, 246
 Tryptophan synthetase
 mutations and, 292, 651-52
 Tryptophanyl-ribonucleic acid synthetase
 specificity of, 302
 Tumors
 vitamin B₆ deficiency and, 404
 Turnip peroxidase
 characterization of, 31
 Turnip yellow mosaic virus
 structure of, 124
 Tyrosine
 ascorbic acid function and, 386-87
 cell culture and, 607
 crystal structure of, 95
 metabolism of, 256-59
 protein nutrition and, 330
 protein spectroscopy and, 284

Tyrosine decarboxylase
 pyridoxal phosphate assay with, 402
 U
 Ubichromenol
 relation to coenzyme Q, 542-43
 Ubiquinol
 antimycin effect on, 22
 Ubiquinone
 biosynthesis of, 542-43
 cytochrome b reduction of, 18
 reactivation of, 22
 tocopherols and, 460
 vitamin A and, 452
 see also coenzyme Q
 Ultraviolet absorption
 of oligonucleotides, 153-55
 Ultraviolet irradiation
 of oligonucleotides, 157
 Uracil
 glycerol derivative of, 297
 Urea
 amino acid metabolism and, 247-48
 ribonuclease optical rotation and, 283
 ribonuclease reduction and, 280-81
 Urea phosphate
 crystal structure of, 115
 Uric acid
 biosynthesis of, 409-10
 pteridine biosynthesis and, 412
 Uridine
 conversion to deoxyuridine, 130
 synthesis of, 137
 Uridine diphosphate-N-acetylglucosamine
 chitin biosynthesis and, 361
 hyaluronic acid synthesis and, 362-63
 Uridine diphosphate galactose
 formation of, 231
 lactose synthesis and, 360
 lipid synthesis and, 360
 Uridine diphosphate-galactose-4-epimerase
 characterization of, 231
 Uridine diphosphate galacturonic acid
 galacturonide synthesis and, 360
 reactions of diphenols with, 360
 Uridine diphosphate glucose
 glycogen synthesis and, 223-24, 362
 hydroxymethyl cytosine glucosylation by, 363

- oligosaccharide synthesis and, 359-60
 - pneumococcal polysaccharide biosynthesis and, 363
 - polysaccharide synthesis and, 361
 - Uridine diphosphate glucuronic acid
 - glucuronide synthesis and, 360
 - hyaluronic acid synthesis, 362-63
 - pneumococcal polysaccharide biosynthesis and, 363
 - Uridine diphosphate sucrose transosylase
 - equilibrium constant of, 365
 - Uridine diphosphate sugars
 - synthesis of, 143-45
 - transosylases and, 359-64
 - Uridine diphosphate xylose
 - pentosan biosynthesis and, 361-62
 - Uridine-5'-pyrophosphate
 - sugar peptides of, 142
 - Uridine triphosphate
 - glycogen synthesis and, 224
 - internal incorporation of, 170
 - Uridylic acid
 - nucleotide sequence and, 168
 - polymerization of, 147
 - Uridyl-5':3'-uridine
 - synthesis of, 149
 - Urinary stones
 - vitamin A and, 453
 - Urine
 - ribonuclease in, 278
 - tocopherol derivatives in, 460-61
 - Uroporphyrin
 - biosynthesis of, 551
 - Uroporphyrinogen
 - biosynthesis of, 551-55
 - Uroporphyrinogen isomerase
 - action of, 554
- V
- Valine
 - crystal structure of, 95
 - isoleucine ribonucleic acid synthetase and, 301-2
 - metabolism of, 250
 - negative feedback and, 246
 - protein nutrition and, 330
 - D-Valine
 - metabolism of, 250
 - Valyl-ribonucleic acids
 - formation of, 300
 - Valyl-ribonucleic acid synthetase
 - specificity of, 301-2
 - Vanadium salts
 - cholesterol synthesis and, 539
 - Venom diesterase
 - action on ribonucleic acids, 301
 - Vernolic acid
 - structure of, 78
 - Viruses
 - polyamines in, 583
 - x-ray diffraction of, 121-25
 - Viscosity
 - of ribonuclease solutions, 282
 - Vitamins
 - ascorbic acid, 383-88
 - biochemistry of, 383-472
 - biotin, 388-91
 - cell culture and, 608
 - fat soluble, 447-72
 - folic acid, 409-27
 - inositol, 391-94
 - nicotinic acid, 394-97
 - riboflavin, 409-27
 - thiamine, 427-35
 - vitamin A, 447-54
 - vitamin B₆, 398-405
 - vitamin B₁₂, 435-41
 - vitamin D, 454-57
 - vitamin E, 458-63
 - vitamin K, 463-66
 - water soluble, 383-446
 - Vitamin A
 - biochemistry of, 447-54
 - determination of, 447-48
 - function of, 450-54
 - kwashiorkor and, 338
 - mevalonic acid metabolism and, 543
 - separation from vitamin D, 454
 - utilization of, 448-50
 - Vitamin A acid
 - utilization of, 450-51
 - Vitamin A aldehydes
 - determination of, 448
 - Vitamin A derivatives
 - interrelationships of, 450
 - Vitamin B₆
 - biochemistry of, 398-405
 - determination of, 402
 - enzymes containing, 398-401
 - metabolic effects, 403-4
 - metabolism of, 401-2
 - nutriture of, 402-3
 - Vitamin B₁₂
 - biochemistry of, 435-41
 - biosynthesis of, 435-37
 - coenzyme form of, 438
 - deoxyriboside synthesis and, 176
 - enzymic reactions of, 439-41
 - isomerizations and, 250
 - mechanism of action, 438-41
 - methionine biosynthesis and, 254-55
 - nucleotide portion of, 436-37
 - porphyrin portion of, 435-36
 - propionate metabolism and, 201-3
 - x-ray diffraction of, 94, 126
 - Vitamin C
 - see Ascorbic acid
 - Vitamin D
 - biochemistry of, 454-57
 - determination of, 454-55
 - function of, 456-57
 - irradiation and, 456
 - utilization of, 455
 - Vitamin E
 - analysis of, 458-59
 - antioxidant relationship, 462-63
 - ascorbic acid synthesis and, 384
 - bioassay of, 459
 - biochemistry of, 458-63
 - chemistry of, 458
 - deficiency symptoms, 20-21, 461-62
 - function of, 459-61
 - ubiquinone reactivation by, 22
 - see also various tocopherols
 - Vitamin K
 - analysis for, 463
 - biochemistry of, 463-66
 - chemistry of, 463
 - enzymatic role of, 464
 - irradiation of, 465
 - oxidative phosphorylation and, 23
 - requirements for, 465
 - ubiquinone reactivation by, 22
 - Vitamin K₃
 - role of, 23
 - Vitamin K reductase
 - purification and properties of, 23
- W
- Wax
 - analysis of, 73
 - Wax D
 - characterization of, 86
 - Wheat
 - vitamin E content of, 459
 - Wheat flour
 - nutritional value of, 337
- X
- Xanthine oxidase
 - biological oxidation and, 36-37

SUBJECT INDEX

753

Xanthopterin
folic acid and, 420

Xanthurenic acid
excretion of, 402

X-ray
wave nature of, 94

X-ray diffraction
of amino acids and peptides, 95-97
of biochemicals, 93-132
of carbohydrates, 125-26
of globular proteins, 102-13

isomorphous replacement
and, 102-4
of myoglobin, 270

of nucleic acids, 113-19
of nucleoproteins, 119-21

of polypeptides and fibrous
proteins, 97-102
of viruses, 121-25

Xylan
acid hydrolysis of, 50
partial methylation of, 65
L-Xylonic acid
biosynthesis of, 388

Xylosanases
biochemistry of, 373

Xylose
pentosan biosynthesis and,
361-62

transport of, 221-22

Y

Yeast

carbohydrate transport
in, 216-17

lipids of, 86-87

protein metabolism and, 332
spermine agglutination of,
600

Z

Zymosterol

metabolism of, 535

CUMULATIVE INDEX

VOLUMES 26 TO 30

INDEX OF CHAPTER TITLES

AMINO ACIDS, PEPTIDES, PROTEINS

Biosynthesis:

H. Chantrenne, 27: 35-56; J. L. Simkin, 28: 145-70; G. N. Cohen and F. Gros, 29: 525-46; P. Berg, 30: 293-324

Chemistry:

D. Steinberg and E. Mihalyi, 26: 373-418; P. Edman, 28: 69-96; R. Schwyzler, 29: 183-206

Metabolism:

H. Kamin and P. Handler, 26: 419-90; M. J. Coon and W. G. Robinson, 27: 561-612; W. E. Knox and E. J. Behrman, 28: 223-56; S. Udenfriend, H. Weissbach and C. Mitoma, 29: 207-60; C. Gilvarg, 30: 239-68

Structure:

R. L. Hill, J. R. Kimmel and E. L. Smith, 28: 97-144; G. E. Perlmann and R. Diring, 29: 151-82; B. S. Harrop, W. B. Gratzer and P. Doty, 30: 269-92

ANTIBIOTICS

E. B. Chain, 27: 167-222

CANCER

H. E. Skipper and L. L. Bennett, Jr., 27: 137-66; E. P. Anderson and L. W. Law, 29: 577-608

Carcinogenesis:

E. C. Miller and J. A. Miller, 28: 291-320

CARBOHYDRATES

Chemistry:

J. C. Sowden, 26: 645-66; R. E. Reeves, 27: 15-34; W. Pigman, K. Nisizawa and S. Tsuiki, 28: 15-38; W. J. Whelan, 29: 105-30; A. B. Foster, 30: 45-70

Metabolism:

C. de Duve and H. G. Hers, 26: 149-80; M. F. Utter, 27: 245-84; H. Holzer, 28: 171-222; A. Beloff-Chain and F. Pocchiari, 29: 295-346; A. Sols, 30: 213-38

CAROTENOIDS

See Pigments, carotenoids

CELLS

Culture of:

L. Levintow and H. Eagle, 30: 605-40

Division of:

D. Mazia, 30: 669-88

CHOLESTEROL

See Steroids, cholesterol

CHROMATOGRAPHY

Gas-Phase:

S. R. Lipsky and R. A. Landowne, 29: 649-68

CLINICAL BIOCHEMISTRY

J. G. Reinhold, 26: 587-610; C. G. Holmberg and R. Blomstrand, 28: 321-42; I. MacIntyre and I. D. P. Wootton, 29: 635-48

CONNECTIVE TISSUE

S. Roseman, 28: 545-78

DRUG AND FOREIGN COMPOUNDS

Metabolism of:

B. B. Brodie, J. R. Gillette and B. N. La Du, 27: 427-54

ENZYMES

Glycosidases and Transglycosidation:

R. A. Dedonder, 30: 347-82

Mechanism:

P. D. Boyer, 29: 15-44

Nonproteolytic and Nonoxidative:

S. Schwimmer, 26: 63-96

Oxygenases and Hydroxylases:

L. Massart and R. Vercauteren, 28: 527-44

Proteolytic:

B. J. Jandorf and H. O. Michel, 26: 97-118; G. H. Dixon, H. Neurath and J.-F.

Pechère, 27: 489-532; B. S. Hartley, 29: 45-72

Transferases:

O. Hoffmann-Ostenhof, 29: 73-104

FISHES

Biochemistry of:

H. L. A. Tarr, 27: 223-44

GENETICS

J. R. S. Fincham, 28: 343-64; R. L. Sinsheimer, 29: 503-24; C. Levinthal and P.

F. Davison, 30: 641-86

HORMONES

Protein:

O. K. Behrens and W. W. Bromer, 27: 57-100; R. Acher, 29: 547-76

Steroid:

See Steroids, hormones

IMMUNOCHEMISTRY

F. Haurowitz, 29: 609-34

Immunopolysaccharides:

See Carbohydrates, immunopolysaccharides

Isoprenoid Compounds:

L. D. Wright, 30: 525-48

LIPIDS

E. Klenk and H. Debusch, 28: 39-68

Chemistry:

J. H. Law, 29: 131-50; J. Asselineau and E. Lederer, 30: 71-92

Metabolism:

E. P. Kennedy, 26: 119-48; P. K. Stumpf, 29: 261-94; R. G. Langdon and A. H.

Phillips, 30: 189-212

METABOLIC ANTAGONISTS

W. Shive and C. G. Skinner, 27: 643-78

MINERAL METABOLISM

See Nutrition, mineral metabolism

MUSCLE

H. H. Weber, 26: 667-98; S. V. Perry, 30: 473-98

NEUROCHEMISTRY

F. N. LeBaron, 28: 579-604; R. H. S. Thompson and G. R. Webster, 29: 365-90

NUCLEIC ACIDS

Nucleotides, Purines and Pyrimidines

W. E. Cohn and E. Volkin, 26: 491-522

Enzymology:

L. A. Heppel and J. C. Rabinowitz, 27: 613-42; C. A. Dekker, 29: 453-74

Metabolism and Biosynthesis:

R. Abrams, 30: 165-88

Purines and Pyrimidines:

L. A. Heppel and J. C. Rabinowitz, 27: 613-42; S. C. Hartman and J. M. Buchanan, 28: 365-410; A. M. Michelson, 30: 133-64

NUTRITION

O. W. Portman and D. M. Hegsted, 26: 307-26; N. S. Scrimshaw, G. Arroyave and

R. Bressani, 27: 403-26; R. E. Olson, 29: 467-98; M. O. Schultze, 29: 391-412;

W. N. Pearson and W. J. Darby, 30: 325-46

Mineral Metabolism:

E. J. Underwood, 28: 499-526

OXIDATIONS, BIOLOGICAL

H. R. Mahler, 26: 17-62; J. B. Neilands, 27: 455-88; M. Klingenberg and T.

Bücher, 29: 669-708; M. Morrison, 30: 11-44

PEPTIDES

See Amino Acids, Peptides, Proteins

PHOSPHATIDES

See Lipids, phosphatides

POLYAMINES

H. Tabor, C. W. Tabor and S. M. Rosenthal, 30: 579-604

PORPHYRINS AND HEMOPROTEINS

C. Rimington, 26: 561-86; E. Margoliash, 30: 549-78

PREFATORY CHAPTERS

R. Peters, 26: 1-16; H. T. Clarke, 27: 1-14; E. F. Terroine, 28: 1-14; H. O. L.

Fischer, 29: 1-14; J. H. Northrop, 30: 1-10

PROTEINS

See Amino Acids, Peptides, Proteins

PURINES AND PYRIMIDINES

See Nucleic Acid, purines and pyrimidines

RUMINANTS

See Nutrition, of ruminants

STEROIDS**Cholesterol:**

G. Popják, 27: 533-60

Hormones:

R. I. Dorfman, 26: 523-60; L. L. Engel and L. J. Langer, 30: 499-524

Metabolism:

P. A. Katzman, E. A. Doisy, Jr., J. T. Matschiner and E. A. Doisy, 28: 257-90

SULFUR COMPOUNDS

J. D. Gregory and P. W. Robbins, 29: 347-64

U.S.S.R.**Biochemistry in:**

J. A. Stekol, 26: 611-44; 27: 679-702; 28: 605-36

VIRUSES

G. Schramm, 27: 101-36; L. M. Kozloff, 29: 475-502

VITAMINS**Fat-Soluble:**

K. L. Baxter, 26: 275-306; S. R. Ames, 27: 371-402; F. Bro-Rasmussen and

W. Hjarde, 30: 447-72

Water-Soluble:

J. R. Totter (Vitamin B₁₂, Folic Acid, Choline, Para-Aminobenzoic Acid), 26: 181-208; L. D. Greenberg (Biotin, Pyridoxin Group, Nicotinamide, Ascorbic Acid), 26: 209-42; G. D. Novelli (Pantothenic Acid, Thiamine, Lipic Acid, Riboflavin, Inositol), 26: 243-74; H. P. Broquist (Folic Acid, B₁₂ Group, Choline), 27: 285-312; C. H. Lushbough and B. S. Schweigert (Ascorbic Acid, Biotin, Nicotinamide, Vitamin B₆), 27: 313-38; H. P. Sarett and A. B. Morrison (Thiamine, Lipic Acid, Pantothenic Acid, Riboflavin, Inositol), 27: 339-70; M. K. Horwitt (Thiamine, Riboflavin, Pantothenic Acid, Nicotinamide, Lipic Acid), 28: 411-38; M. E. Coates and J. W. G. Porter (Vitamin B₁₂, Folic Acid, Ascorbic Acid, Biotin, Vitamin B₆, Miscellaneous), 28: 439-66; J. J. Burns and A. H. Conney (Ascorbic Acid, Nicotinic Acid, Vitamin B₆, Biotin, Inositol), 29: 413-36; J. S. Dinning (Vitamin B₁₂, Folic Acid, Thiamine, Riboflavin, Pantothenic Acid), 29: 437-52; A. E. Axelrod and C. J. Martin (Water-Soluble Vitamins, Part I), 30: 383-408; G. W. E. Plaut (Water-Soluble Vitamins, Part II), 30: 409-46

X-RAY STUDIES OF STRUCTURE

J. C. Kendrew and M. F. Perutz, 26: 327-72; A. Rich and D. W. Green, 30: 93-132



INDEX OF CONTRIBUTING AUTHORS

- | | | |
|---|--|---|
| <p>A</p> <p>Abrams, R., 30: 165
Acher, R., 29: 547
Ames, S. R., 27: 371
Anderson, E. P., 29: 577
Arroyave, G., 27: 403
Asselineau, J., 30: 71
Axelrod, A. E., 30: 383</p> <p>B</p> <p>Behrens, O. K., 27: 57
Behrman, E. J., 28: 223
Beloff-Chain, A., 29: 295
Bennett, L. L., Jr., 27: 137
Berg, P., 30: 293
Blaxter, K. L., 26: 275
Blomstrand, R., 28: 321
Bressani, R., 27: 403
Brodie, B. B., 27: 427
Bromer, W. W., 27: 57
Broquist, H. P., 27: 285
Bro-Rasmussen, F., 30: 447
Buchanan, J. M., 28: 365
Bücher, T., 29: 669
Burns, J. J., 29: 413</p> | <p>C</p> <p>Chain, E. B., 27: 167
Chantrenne, H., 27: 35
Clarke, H. T., 27: 1
Coates, M. E., 28: 439
Cohen, G. N., 29: 525
Cohn, W. E., 26: 491
Conney, A. H., 29: 413
Coon, M. J., 27: 561</p> <p>D</p> <p>Darby, W. J., 30: 325
Davison, P. F., 30: 641
Debueh, H., 28: 39
Dedonder, R. A., 30: 347
de Duve, C., 26: 149
Dekker, C. A., 29: 453
Dinning, J. S., 29: 437
Diringer, R., 29: 151
Dixon, G. H., 27: 489
Doisy, E. A., 28: 257
Doisy, E. A., Jr., 28: 257
Dorfman, R. I., 26: 523
Doty, P., 30: 269</p> <p>E</p> <p>Eagle, H., 30: 605
Edman, P., 28: 69</p> | <p>Engle, L. L., 30: 499</p> <p>F</p> <p>Fincham, J. R. S., 28: 343
Fischer, H. O. L., 29: 1
Foster, A. B., 30: 45</p> <p>G</p> <p>Gillette, J. R., 27: 427
Gilvarg, C., 30: 239
Glatzer, W. B., 30: 269
Green, D. W., 30: 93
Greenberg, L. D., 26: 209
Gregory, J. D., 29: 347
Gros, F., 29: 525</p> <p>H</p> <p>Handler, P., 26: 419
Harrap, B. S., 30: 269
Hartley, B. S., 29: 45
Hartman, S. C., 28: 365
Haurowitz, F., 29: 609
Hegsted, D. M., 26: 307
Heppel, L. A., 27: 613
Hers, H. G., 26: 149
Hill, R. L., 28: 97
Hjarde, W., 30: 447
Hoffmann-Ostenhof, O.,</p> |
|---|--|---|

29: 73
Holmberg, C. G., 28: 321
Holzer, H., 28: 171
Horwitz, M. K., 28: 411

J

Jandorf, B. J., 26: 97

K

Kamin, H., 26: 419
Katzman, P. A., 28: 257
Kendrew, J. C., 26: 327
Kennedy, E. P., 26: 119
Kimmel, J. R., 28: 97
Klenk, E., 28: 39
Klingenberg, M., 29: 669
Knox, W. E., 28: 223
Kozloff, L. M., 29: 475

L

La Du, B. N., 27: 427
Landowne, R. A., 29: 649
Langdon, R. G., 20: 189
Langer, L. J., 30: 499
Law, J. H., 29: 131
Law, L. W., 29: 577
LeBaron, F. N., 28: 579
Lederer, E., 30: 71
Levinthal, C., 30: 641
Levitow, L., 30: 605
Lipsky, S. R., 29: 649
Lushbough, C. H., 27: 313

M

MacIntyre, I., 29: 635
Mahler, H. R., 26: 17
Margoliash, E., 30: 549
Martin, C. J., 30: 383
Massart, L., 28: 527
Matschner, J. T., 28: 257
Mazia, D., 30: 669
Michel, H. O., 26: 97
Michelson, A. M., 30: 133
Mihalyi, E., 26: 373
Miller, E. C., 28: 291

Miller, J. A., 28: 291
Mitoma, C., 29: 207
Morrison, A. B., 27: 339
Morrison, M., 30: 11

N

Neillands, J. B., 27: 455
Neurath, H., 27: 489
Nisizawa, K., 28: 15
Novelli, G. D., 26: 243
Northrop, J. H., 30: 1

O

Olson, R. E., 28: 467

P

Pechère, J.-F., 27: 489
Perlmann, G. E., 29: 151
Pearson, W. N., 30: 325
Perry, S. V., 30: 473
Perutz, M. F., 26: 327
Peters, R., 26: 1
Phillips, A. H., 30: 189
Pigman, W., 28: 15
Plaut, G. W. E., 30: 409
Pocchiari, F., 29: 295
Popják, G., 27: 533
Porter, J. W. G., 28: 439
Portman, O. W., 26: 307

R

Rabinowitz, J. C., 27: 613
Reeves, R. E., 27: 15
Reinhold, J. G., 26: 587
Rich, A., 30: 93
Rimington, C., 26: 561
Robbins, P. W., 29: 347
Robinson, W. G., 27: 561
Roseman, S., 28: 545
Rosenthal, S. M., 30: 579

S

Sarett, H. P., 27: 339
Schramm, G., 27: 101

Schultze, M. O., 29: 391
Schweigert, B. S., 27: 313
Schwimmer, S., 26: 63
Schwyzer, R., 29: 183
Scrimshaw, N. S., 27: 403
Shive, W., 27: 643
Simkin, J. L., 28: 145
Sinsheimer, R. L., 29: 503
Skinner, C. G., 27: 643
Skipper, H. E., 27: 137
Smith, E. L., 28: 97
Sols, H., 30: 213
Sowden, J. C., 26: 645
Steinberg, D., 26: 373
Stekol, J. A., 26: 611;
27: 679; 28: 605
Stumpf, P. K., 29: 261

T

Tabor, C. W., 30: 579
Tabor, H., 30: 579
Tarr, H. L. A., 27: 223
Terroine, E. F., 28: 1
Thompson, R. H. S., 29: 365
Totter, J. R., 26: 181
Taulki, S., 26: 15

U

Udenfriend, S., 29: 207
Underwood, E. J., 28: 499
Utter, M. F., 27: 245

V

Vercauteren, R., 28: 527
Volkin, E., 26: 491

W

Weber, H. H., 26: 667
Webster, G. R., 29: 365
Weissbach, H., 29: 207
Whelan, W. J., 29: 105
Wootton, I. D. P., 29: 635
Wright, L. D., 30: 525

